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Mutational Analysis of Oil Degrading Genes in Bacterial Isolates from Oil Contaminated Soil at the Jordanian Oil Refinery

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Abstract: Petroleum hydrocarbons are major contaminants in the environment, as they cause damages to the surrounding ecosystems. Removal of those contaminants is becoming a very important problem. The current study aimed to create more effective oil-degrading microorganisms by inducing random mutations using UV irradiations onto hydrocarbons-degrading key enzymes (catechol 2, 3 dioxygenase (C23O) and monooxygenase) into thirteen locally isolated oil-degrading bacterial isolates and testing their salt tolerance ability, to be used in cleaning oil spills in oil contaminated soil sites and in high saline environments. Two UV irradiated bacterial isolates (*Pseudomonas aeruginosa* TDJ2^{M} and *Pseudomonas putida* TDJ6^{M}) showed an increase of C23O enzyme activity, compared to their wild-type isolates and one mutant bacterial isolate (*Pseudomonas mallei* TDJ4^{M}) showed an increase in monooxygenase activity, compared to its wild-type isolate. Five bacterial isolates were able to grow at NaCl concentration up to (8% and 10%), 4 bacterial isolates were able to grow at NaCl concentrations up to (12 %, 14 %, 16 %, 18 % and 20 %), while *Moraxella sp.* TDJ9 was the only isolate able to grow up to 25 % NaCl concentration and therefore could be recommended to be used as seed microbes in hyper-saline environments. RAPD analysis demonstrated its ability to reveal genome differences and polymorphic bands between the wild-type and their UV irradiated mutant isolates.

Key words: UV irradiation · Oil biodegradation · Bacteria · Soil

INTRODUCTION

Oil spills are major hazards to the environment as they damage the surrounding ecosystems [1]. Petroleum fuel spills as a result of pipeline ruptures, tank failure, various production storage and transportation accidents are considered as the most frequent organic pollutant of soil and aquatic environment and are classified as hazardous wastes due to their cytotoxic, mutagenic and carcinogenic effects on human [2, 3].

The search for efficient and effective methods of oil removal from contaminated soils has intensified in recent years. One promising method that has been studied is the biological degradation of oil by microbes [4, 5]. Microorganisms are equipped with metabolic machinery to use petroleum as a carbon and energy sources [6, 7] They are involved in degrading a considerable number of organic pollutants that are involved in oil including: aliphatic compounds, n-alkanes, diesel fuel and tetrachloroethylene, monoaromatic compounds, toluene, benzene, xylene, ethylbenzene and polycyclic aromatic hydrocarbons [8, 10]. The identification of key organisms that play a role in pollutant degradation processes is relevant to the development of optimal in situ bioremediation strategies [11, 12].

The interest in discovering how bacteria are dealing with hazardous environmental pollutants has driven a large research community and has resulted in important biochemical, genetic and physiological knowledge about the degradation capacities of microorganisms and their applications in bioremediation [7, 13].

Bioremediation is a popular approach of cleaning up petroleum hydrocarbons because it is simple to maintain, leads to the destruction of contaminants, applicable over large areas and cost effective [4]. Different genera of bacteria are known for their potential oil degradation in which they contain different degradative enzymes involved in the metabolism of hydrocarbons [14, 15]. There are seven catabolic genes that encode enzymes involved in a variety of known bacterial hydrocarbon degradative pathways: *alkB*; alkane monooxygenase from *Pseudomonas putida*, formerly designed *P. oleovorans*

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(C_5 to C_{12} alkane degradation); *alkm*; alkane monooxygenase from *Acinetobacter* sp. strain ADP-1 (C_{10} to C_{20} alkane degradation); *alkB1* and *alkB2* (C_{12} to C_{16} alkane degradation), alkane monooxygenase from *Rhodococcus* spp.; *xylE*, catechol-2, 3-dioxygenase from *P. putida* (xylene and toluene degradation); *ndoB*; naphthalene dioxygenase from *P. putida* (PAH; naphthalene degradation); and *nidA*; pyrene dioxygenase large subunit from *Mycobacterium* sp. strain PYR-1, (PAH; pyrene degradation) [2, 6].

Because of their importance in degradation of aromatic rings, extradiol dioxygenases have received a lot of attention. In fact, the first dioxygenase discovered was catechol 1, 2-dioxygenase, which cleaves the aromatic ring of catechol [16]. The substrates of the ring-cleavage dioxygenases usually contain two hydroxyl groups on two adjacent aromatic carbons and ring-cleavage dioxygenases can be classified into two groups according to the mode of scission of the aromatic ring. Intradiol enzymes cleave the aromatic ring between two hydroxyl groups, whereas extradiol enzymes, such as catechol 2, 3 dioxygenase, cleave the aromatic ring between one hydroxylated carbon and another adjacent non-hydroxylated carbon. These enzymes play a key role in the metabolism of aromatic compounds. In bacteria, they are responsible for the cleavage of aromatic rings during aerobic catabolism of compounds such as toluene and xylene, naphthalene or biphenyl derivatives, some of which are environmental pollutants of serious concern [16]. Consequently, these enzymes were the subject of different studies and have been suitable targets for site directed mutagenesis to improve their activities by selecting the best mutant which had the best activity in oil degradation and use them in future for cleaning oil spills in the environment [17].

Recently thirty-four oil-degrading bacteria have been isolated from soil sites in a refinery station in Jordan that had been contaminated with petroleum oil [8].

In this current work, we aimed to do further characterization such as salt tolerance of some selected bacterial isolates from the above study, so they could be adapted to oil biodegradation in the marine environment and in brine-soil contaminated with hydrocarbons and to further induce random mutations into those bacterial isolates to select the UV irradiated counterpart isolates with highest catechol 2, 3 dioxygenase (C23O) activity and Alkane monooxygenase activity, then further characterize them genetically by using PCR based molecular approach.

MATERIALS AND METHODS

Bacterial Strains and Chemicals: Thirteen bacterial isolates (Table 1) were isolated by Al-Deeb [8] from 40 oil-contaminated soil samples, which were collected from several locations at the Jordanian oil refinery in Zarqa (Northeast of Jordan). All isolates were isolated according to their ability to degrade oil and were identified to belong to several genera and species. Each of the selected thirteen bacterial isolates was sub-cultured in nutrient agar supplemented with 0.1 % crude oil and incubated at 25°C for 3 days. Pure colonies of sub-cultured bacterial isolates were stored in stab culture, kept in the refrigerator in glycerol broth and kept at -70°C for analysis.

All chemicals, media and reagents were purchased from Sigma Chemicals Co. USA unless otherwise specified, as below: Agarose, Proteinase K enzyme, Ribonuclease enzyme (Rnase), PCR reagents Kit [10X PCR reaction buffer, 25mM $MgCl_2$, 10mM deoxy-nucleotides (dNTPs), Taq-DNA polymerase enzyme and nuclease-free water] (were from Promega Co. Madison. USA), all media for bacteria were purchased from (HiMedia Laboratories Ltd. India), primers for PCR reactions were from (Alpha DNA Quebec. Canada), 2, 6-Dichlorophenol-indophenol (DCPIP), 5-Methyl-phenazinummethylsulphate (MPMS), Tris HCl ñ-Dimethyl-aminobenzaldehyde (from Acros Organics USA), di-Potassium hydrogen phosphate (K_2HPO_4), Glucose, Sodium hydrogen phosphate ($NaHPO_4$), di-Sodium hydrogen phosphate (Na_2HPO_4) and, Lactose (from PS. Park. Ltd. UK), â-Nicotinamide adenine dinucleotide (NAD) (from AppliChem. GmbH. Germany), Ethanol, Ferrous chloride ($FeCl_3$) and Sodium molybdate ($NaMoO_4$) (from BDH Chemicals Ltd. UK).

Salt Tolerance Test: All the thirteen oil degrading bacterial isolates were assayed to grow on different sodium chloride (NaCl) concentrations (3, 5, 8, 10, 12, 14, 16, 18, 20, 25 and 28 %) and were grown also on an artificial seawater mineral salts medium ONR7 for testing salt growth ability.

Mutagenesis Using Uv Irradiation of Oil-degrading Bacterial Isolates: Plates with 100 µl of each overnight bacterial isolate were placed under the UV lamp (16 W) at two different distances. The exposure time of the bacteria to the UV light was: 0, 5, 10, 20, 30, 45, 60 sec. at 15 cm from UV lamps and 10, 20, 30 sec. at 30 cm of distance. After each exposure time, the plate was incubated at 37°C

Table 1: Morphological characteristics of the 13 oil degrading bacterial isolates cultured on nutrient agar medium

Isolates	Colony color	Colony size	Colony form	Colony elevation	Colony margin	Gram stain
Pseudomonas aeruginosa TDJ2	Blue green	Medium	Irregular	Raised	Undulated	Gram -ve Coccobacilli
Bacillus megaterium TDJ3	Beige-brown	Medium	Irregular	Raised slightly	Undulated	Gram +ve Rod
P. mallei TDJ4	Beige	Large	Circular	Convex	Entire	Gram -ve Coccobacilli
Acinetobacter calcoaceticus TDJ5	Orange	Medium	Irregular	Slightly elevated	Convex	Gram -ve Coccobacilli
P. putida TDJ6	Brown	Medium	Circular	Flat	Entire	Gram -ve Coccobacilli
Moraxella sp. TDJ9	Beige	Small	Punctiform	Convex	Entire	Gram -ve Cocci
Comamonas sp. TDJ10	Beige-yellow	Medium	Circular	Slightly raised	Arose	Gram -ve Rod
Micrococcus roseus TDJ19	Pink-red	Small	Circular	Pulvinate	Entire	Gram +ve Cocci
P. stutzeri TDJ20	Yellow	Medium	Filamentous	Raised	Undulated	Gram -ve Coccobacilli
P. stutzeri TDJ24	Yellow	Medium	Circular	Slightly raised	Entire	Gram -ve Coccobacilli
P. mallei TDJ29	Light-yellow	Medium	Circular	Slightly raised	Entire	Gram -ve Coccobacilli
Bordetella sp. TDJ31	Beige	Large	Irregular	Flat	Undulated	Gram -ve Coccobacilli
P. oleovorans TDJ34	Beige-yellow	Medium	Filamentous	Slightly raised	Lobated	Gram -ve Coccobacilli

-ve: Indicate Gram negative, +ve: Indicate Gram positive.

for 24 h. The number and morphology of colonies on each plate were recorded. The effect of UV irradiation on C23O and alkane monooxygenase activities was tested using enzyme assay protocols.

Catechol 2, 3 Dioxygenase Enzyme Assay

Catechol Oxidation: Five *Pseudomonas* bacterial isolates and their UV irradiated colonies (*P. aeruginosa* TDJ2 and TDJ2^{M}, *P. putida* TDJ6 and TDJ6^{M}, *P. stutzeri* TDJ20 and TDJ20^{M}, *P. stutzeri* TDJ24 and TDJ24^{M}, *P. mallei* TDJ29 and TDJ29^{M}) were grown on Tryptic Soy Agar (TSA) for 48 h and then resuspended in 50 mM phosphate buffer, pH 7.4, to a cell density of 1 optical density at 600 nm (OD600) unit, which corresponds to 10^{9} CFU/ml. Reactions were initiated by adding cells (1 ml) to reaction mixtures containing 10 mM catechol in 50 mM potassium phosphate, pH 7.4. Catechol oxidation to 2-hydroxymuconic semialdehyde was monitored continuously at 375 nm with a spectrophotometer at room temperature. A unit of C23O activity corresponds to 1 µmol of catechol/min and values were expressed as units per minute per 10^{8} cells [18].

Tryptic Soy Agar (TSA) Test Before and after UV Irradiation: Wild-type bacterial isolates and their UV irradiated counterparts were incubated for three days into TSA media and then stored overnight at 4°C. The following day, the plates were sprayed with 0.5M catechol in 50mM potassium phosphate (pH 7.5) to distinguish the xylE-expressing colonies, which become yellow (storing plates overnight at 4°C results in stronger yellow color) [19].

Alkane Monooxygenase Assay: Wild-type bacterial isolates and their UV irradiated counterparts were tested for their diesel degradation ability after UV irradiation at 15 cm distance from UV lamp after (10, 20 and 30s) and at 30 cm distance after (20 and 30s). For UV irradiated bacteria comparing to their wild-types, alkane monooxygenase test was done as described by Al-Deeb [8].

Molecular Characterization

Dna Extraction and Pcr Conditions: Extraction of genomic DNA from the thirteen bacterial isolates was done using Wizard® Genomic DNA purification kit according to the manufacturer instructions (Promega, USA). Total genomic DNA isolated from each bacterial isolate was subjected to PCR amplification using the primer pair specific to the degrdative gene catechol 2, 3 dioxygenase (their 5'-3'sequences are: DEG-Forward: CGACCTGATCATCGCAT GACCGA and DEG-Reverse: TCTAGGTCAGTACACGGT CA) according to Mesarch *et al*. [9].

RAPD amplification was performed in a final volume of 25 µl containing 10X PCR buffer, 2 mM $MgCl_2$, 100 µM each of dNTPs, 25 ng genomic DNA, 0.5 units of *Taq* polymerase and 0.5 µM of each primer. Three random primers (Operon Techn. Inc. Alameda, CA) were used and their sequences (5'-3') are as follow: OPA3 (AGTCAGCCAC) OPA9 (GGGTAACGCC) and OPA18 (AGGTGACCGT). Reactions were performed in a thermocycler (Perkin Elmer 480). A total of 45 cycles of amplification were performed with template DNA denaturation at 94°C for 1 min, primer annealing at 35°C

for 1 min and primer extension at 72°C for 2 min. PCR products were separated using 1.5% agarose gel electrophoresis and visualized with 0.5 µg/ml ethidium bromide staining. The sizes of the fragments were estimated based on a DNA ladder of 1K bp.

RESULTS

Salt Tolerance of the Bacterial Isolates and Their UV-MUTANTS: At NaCl concentrations 3 and 5%, the wild-type bacterial isolates and their UV-mutants were able to grow, except for (*Comamonas sp.* TDJ10 and TDJ10^{M}, *P. stutzeri* TDJ24 and TDJ24^{M}) which were able to grow at 5% NaCl concentration.

At NaCl concentrations of 8 and 10%, bacterial isolates (*P. mallei* TDJ4 and TDJ4^{M}, *Acinetobacter calcoaceticus* TDJ5 and TDJ5^{M}, *Moraxella sp.* TDJ9 and TDJ9^{M}, *P. mallei* TDJ29 and TDJ29^{M} and *P. oleovorans* TDJ34 and TDJ34^{M}) were able to grow, while the rest of bacterial isolates were not able to grow.

At NaCl concentrations: 12, 14, 16, 18 and 20%, only four bacterial isolates and their UV-mutants (*P. mallei* TDJ4 and TDJ4^{M}, *Moraxella sp.* TDJ9 and TDJ9^{M}, *P. mallei* TDJ29 and TDJ29^{M} and *P. oleovorans* TDJ34 and TDJ34^{M}) were able to grow. Only one bacterial isolate (*Moraxella sp.* TDJ9) was able to grow at 25% NaCl concentration, which makes it the highest salt tolerant isolate. At 28% NaCl concentration, none of the 13 bacterial isolates was able to grow, while *Moraxella sp.* TDJ9^{M} was able to grow after 24 h incubation instead of 72 h as compared to the wild-type bacterial isolate at 25% NaCl concentration.

Bacterial isolates (*Pseudomonas aeruginosa* TDJ2, *Bacillus megaterium* TDJ3, *Acinetobacter calcoaceticus* TDJ5, *P. putida* TDJ6, *Comamonas sp.* TDJ10, *Micrococcus roseus* TDJ19, *P. stutzeri* TDJ20, *P. stutzeri* TDJ24 and *Bordetella sp.* TDJ31, didn't show any morphological differences due to NaCl concentrations used (3 and 5%), that was also the case of their UV irradiated counterparts, which didn't show morphological differences and gave the same morphology with their wild-types. On the other hand, bacterial isolates with the ability to grow at NaCl concentrations higher than 5%, showed differences in colony or media color, in which was the case of *P. mallei* TDJ4 and TDJ4^{M}, *Moraxella sp.* TDJ9 and TDJ9^{M}, *P. mallei* TDJ29 and TDJ29^{M} and *P. oleovorans* TDJ34 and TDJ34^{M} (data not shown), showing differences at NaCl concentration varying from 12 to 20%, of media color, from beige to beige white (12, 14 and 16%) and to beige brown (18 and 20%). As indicated, the UV irradiated counterpart isolates showed the same salt tolerance results, same morphology and color pigmentation as the wild-type isolates. Table (2) summarizes the salt tolerance test results of the 13 bacterial isolates under study.

The Ability to Grow on the Onr7 Salt Medium: An artificial seawater mineral salt medium (ONR7) based on the ionic composition of seawater with NaCl concentration of 3% was used also in this study. All bacterial isolates were grown on ONR7 media for testing their salt growth ability on this medium. The bacterial isolates; *P. mallei* TDJ4, *Moraxella sp.* TDJ9, *P. mallei* TDJ29 and *P. oleovorans* TDJ34 were able to grow in

Table 2: Salt tolerance of the 13 bacterial isolates (wild-types and their UV irradiated counterparts) after four days incubation at 28°C

	Concentrations of NaCl (%)										
Bacterial isolates	3	5	8	10	12	14	16	18	20	25	28
Pseudomonas aeruginosa TDJ2 and TDJ2^{M}	+	+	-	-	-	-	-	-	-	-	-
Bacillus megaterium TDJ3 and TDJ3^{M}	+	+	-	-	-	-	-	-	-	-	-
P. mallei TDJ4 and TDJ4^{M}	+	+	+	+	+	+	+	+	+	-	-
Acinetobacter calcoaceticus TDJ5 and TDJ5^{M}	+	+	+	+	-	-	-	-	-	-	-
P. putida TDJ6 and TDJ6^{M}	+	+	-	-	-	-	-	-	-	-	-
Moraxella sp. TDJ9 and TDJ9^{M}	+	+	+	+	+	+	+	+	+	+	-
Comamonas sp. TDJ10 and TDJ10^{M}	+	-	-	-	-	-	-	-	-	-	-
Micrococcus roseus TDJ19 and TDJ19^{M}	+	+	-	-	-	-	-	-	-	-	-
P. stutzeri TDJ20 and TDJ20^{M}	+	+	-	-	-	-	-	-	-	-	-
P. stutzeri TDJ24 and TDJ24^{M}	+	-	-	-	-	-	-	-	-	-	-
P. mallei TDJ29 and TDJ29^{M}	+	+	+	+	+	+	+	+	+	-	-
Bordetella sp. TDJ31 and TDJ31^{M}	+	+	-	-	-	-	-	-	-	-	-
P. oleovorans TDJ34 and TDJ34^{M}	+	+	+	+	+	+	+	+	+	-	-

(+) indicates presence of growth, (-) indicates absence of growth.

Table 3: Catechol 2, 3 dioxygenase activities of bacterial isolates before and after UV irradiation

		Bacterial isolates				
		*Activity (λmol/ml/mn/10^8cells)				
Time exposureto UV irradiation		*P. aeruginosa* TDJ2 and TDJ2^M (5-60s and 10-30s)	*P. putida* TDJ6 and TDJ6^M (5-60s and 10-30s)	*P. stutzeri* TDJ20 and TDJ20^M (5-60s and 10-30s)	*P. stutzeri* TDJ24 and TDJ24^M (5-60s and 10-30s)	*P. mallei* TDJ29 and TDJ29^M (5-60s and 10-30s)
15 cm						
Wild-type		0.136	0.164	0.129	0.179	0.100
UV irradiated	5s	0.193	0.271	0.079	0.079	0.114
Counterparts	10s	0.293	0.271	0.000	0.007	0.286
	20s	0.307	0.064	0.114	0.100	0.286
	30s	4.300	0.257	0.000	0.200	0.150
	45s	0.307	0.293	0.164	0.036	0.207
	60s	0.207	0.107	0.057	0.000	0.200
30cm						
UV irradiated	10s	0.200	0.129	0.136	0.071	0.129
Counterparts	20s	0.293	0.300	0.121	0.064	0.100
	30s	0.507	3.400	0.179	0.057	0.150

Numbers in bold indicate highest enzyme activity in each isolate.

15cm and 30cm: indicate distance of UV lamp from plate cover.

*A unit of C23O activity corresponds to 1 µmol of catechol/ml and values were expressed as units per minute per 10^8 cells. Activity (Unit/mn/10^8cells) = [(ÄA_{375}/absorption coefficient) x df]/time, where df is the dilution factor and absorption coefficient of 2-hydroxymuconic semialdehyde is 42000 $mol^{-1}cm^{-1}$ at 375nm and pH=7.4.

ONR7 medium, after 3 days of incubation at 28°C, while no visible growth was seen before this time. The rest of the thirteen bacterial isolates (*Pseudomonas aeruginosa* TDJ2, *Bacillus megaterium* TDJ3, *Acinetobacter calcoaceticus* TDJ5, *P. putida* TDJ6, *Comamonas sp* TDJ10, *Micrococcus roseus* TDJ19, *P. stutzeri* TDJ20, *P. stutzeri* TDJ24 *and Bordetella sp.* TDJ31 were not able to grow on this medium. The obtained results confirmed the previous NaCl test, where only *P. mallei* TDJ4, *Moraxella sp.* TDJ9, *P. mallei* TDJ29 and *P. oleovorans* TDJ34 were able to grow at 20% NaCl concentration.

Mutagenesis of Bacterial Isolates by Uv Irradiation: After UV irradiation, no morphological changes were seen in the bacterial isolates comparing to their wild-type isolates. Knowing that, the used UV intensities applied at different times were not lethal to the bacterial growth.

Catechol 2, 3-dioxygenase Activities Before and after Uv Irradiation: The activities were measured in each isolate before and after UV irradiation, the results showed that the higher enzyme activity (Table 3). The UV irradiated *Pseudomonas aeruginosa* TDJ2^M was obtained after 30s of exposure at 15 cm distance from the UV lamp, which was 30 times higher in C23O activity (4.3 λmol/ml/mn/10^8cells) than its wild-type bacterial isolate *Pseudomonas aeruginosa* TDJ2 (0.136 λmol/ml/mn/10^8cells) C23O activity.

The UV irradiated *P. putida* TDJ6^M was obtained after 30s of exposure at 30 cm distance from the UV lamp, which was 20 times higher in C23O activity (3.4 λmol/ml/mn/10^8cells) than its wild-type bacterial isolate *P. putida* TDJ6 (0.164 λmol/ml/mn/10^8cells) C23O activity.

The UV irradiated *P. stutzeri* TDJ20^M was obtained after 30s of exposure at 30 cm distance from the UV lamp, which was 1.4 times higher in C23O activity (0.179 λmol/ml/mn/10^8cells) than its wild-type bacterial isolate *P. stutzeri* TDJ20 (0.129 λmol/ml/mn/10^8cells) C23O activity.

The UV irradiated *P. stutzeri* TDJ24^M was obtained after 30s of exposure at 15 cm distance from the UV lamp, which was 1.1 times higher in C23O activity (0.2 λmol/ml/mn/10^8cells) than its wild-type bacterial isolate *P. stutzeri* TDJ24 (0.179 λmol/ml/mn/10^8cells) C23O activity. The UV irradiated *P. mallei* TDJ29^M was obtained after 10s and 20s of exposure at 15 cm distance from the UV lamp and was 2.9 times higher in C23O activity

Table 4: Morphological characteristics of bacterial isolates (grown on TSA media for 3 days) after UV irradiation

(A)					
P. stutzeri TDJ20 and TDJ2^{M}		Colony color (Surface view)	Colony color (Bottom view)	Media color	Colony shape
Wild-type		light yellow	Beige white	beige	Filamentous
15cm					
UV irradiated	5s	light yellow	Beige	beige	Filamentous
counterparts	10s	light yellow	Beige	beige	Filamentous
	20s	light yellow	Beige	beige	Filamentous
	30s	light yellow	Beige	beige	Filamentous
	45s	light yellow	Beige	beige	Filamentous
	60s	light yellow	Beige	beige	Filamentous
30cm					
UV irradiated	10s	light yellow	Beige	beige	Filamentous
counterparts	20s	light yellow	Beige	beige	Filamentous
	30s	light yellow	Beige	beige	Filamentous
(D)					
P. stutzeri TDJ24 and TDJ24^{M}		Colony color (Surface view)	Colony color (Bottom view)	Media color	Colony shape
15cm					
Wild-type	light yellow	Beige	beige	circular nucleated	
UV irradiated	5s	light yellow	Beige	beige	circularnucleated
counterparts	10s	light yellow	Beige	beige	circularnucleated
	20s	light yellow	Beige	beige	circularnucleated
	30s	light yellow	Beige	Beige brown	circularnucleated
	45s	light yellow	Beige	Beige brown	circularnucleated
	60s	light brown	Beige green	beige	circularnucleated
30cm					
UV irradiated	10s	light yellow	Beige	beige	circularnucleated
counterparts	20s	light yellow	Beige	beige	circularnucleated
	30s	light yellow	Beige	beige	circularnucleated
(E)					
P. mallei TDJ29 and TDJ29^{M}		Colony color (Surface view)	Colony color (Bottom view)	Media color	Colony shape
15cm					
Wild-type	light yellow	Beige	beige	Circular	
UV irradiated	5s	light yellow	Beige	beige	Circular
counterparts	10s	light green	beige brown	brown	Circular
	20s	light green	beige brown	brown	Circular
	30s	light yellow	Beige	beige	Circular
	45s	light green	beige brown	brown	Circular
	60s	light green	beige brown	brown	Circular
30cm					
UV irradiated	10s	light yellow	Beige	beige	Circular
counterparts	20s	light yellow	Beige	beige	Circular
	30s	light yellow	Beige	beige	circular

(0.286 λmol/ml/mn/10^{8}cells) than its wild-type bacterial isolate *P. mallei* TDJ29 (0.1 λmol/ml/mn/10^{8}cells) C23O activity.

As observed, the highest enzyme activities were obtained respectively in *Pseudomonas aeruginosa* TDJ2^{M} (30 times higher than the control) and *Pseudomonas putida* TDJ6^{M} (20 times higher than the control). No major differences in enzyme activities were shown between the UV irradiated counterparts and wild-type isolates for the rest of the bacterial isolates (Table 3).

Tryptic Soy Agar (Tsa) Test to Confirm C23o Activity: UV irradiated bacterial isolates were also subjected to TSA test to check for the activity of C23O in the mutants. TSA test can confirm the presence or absence of C23O activity indicated by the color change of colonies and media to yellow color after the addition of catechol as substrate for C23O. TSA test has confirmed the presence of C23O activity in all control bacterial isolates (*Pseudomonas aeruginosa* TDJ2, *P. putida* TDJ6, *P. stutzeri* TDJ20, *P. stutzeri* TDJ24 and *P. mallei* TDJ29)

Table 5: Alkane monooxygenase activity of the wild-type and their UV irradiated counterpart bacterial isolates after exposure to UV irradiations for: 2, 6, 8, 12, 24 and 72h

UV irradiation times		2h	6h	8h	12h	24h	72h
Pseudomonas aeruginosa TDJ2 and TDJ2^{M}	Wild-type TDJ2 TDJ2^{M}	+	+	+	+	+	+
	30s (15cm)	+	+	+	+	+	+
	60s (15cm)	+	+	+	+	+	+
	20s (30cm)	+	+	+	+	+	+
	30s (30cm)	+	+	+	+	+	+
Bacillus megaterium TDJ3 and TDJ3^{M}	Wild-type TDJ3 TDJ3^{M}	+	+	+	+	+	+
	30s (15cm)	-	-	-	-	-	-
	60s (15cm)	-	-	-	-	-	-
	20s (30cm)	-	-	-	-	-	-
	30s (30cm)	-	-	-	-	-	-
P. mallei TDJ4 and TDJ4^{M}	Wild-type TDJ4 TDJ4^{M}	+	+	+	+	+	+
	30s (15cm)	+	+	+	+	+	+
	60s (15cm)	+	+	+	+	+	+
	20s (30cm)	+	+	+	++	++	++
	30s (30cm)	+	+	+	++	++	++
Acinetobacter calcoaceticus TDJ5 and TDJ5^{M}	Wild-type TDJ5 TDJ5^{M}	+	+	+	+	+	+
	30s (15cm)	-	-	-	+	+	+
	60s (15cm)	-	-	+	+	+	+
	20s (30cm)	-	-	-	-	-	-
	30s (30cm)	-	-	+	+	+	+
Moraxella sp. TDJ9 and TDJ9^{M}	Wild-type TDJ9 TDJ9^{M}	-	-	-	-	-	-
	30s (15cm)	-	-	-	-	-	-
	60s (15cm)	-	-	-	-	-	-
	20s (30cm)	-	-	-	-	-	-
	30s (30cm)	-	-	-	-	-	-
Comanamas sp. TDJ10 and TDJ10^{M}	Wild-type TDJ10 TDJ10^{M}	+	+	+	+	+	+
	30s (15cm)	-	-	-	-	-	-
	60s (15cm)	-	-	-	-	-	-
	20s (30cm)	-	-	-	-	-	-
	30s (30cm)	-	-	-	-	-	-
Micrococcus roseus TDJ19 and TDJ19^{M}	Wild-type TDJ19 TDJ19^{M}	+	+	+	+	+	+
	30s (15cm)	+	+	+	+	+	+
	60s (15cm)	+	+	+	+	+	+
	20s (30cm)	+	+	+	+	+	+
	30s (30cm)	+	+	+	+	+	+
P. stutzeri TDJ24 and TDJ24^{M}	Wild-type TDJ24 TDJ24^{M}	-	-	-	+	+	+
	30s (15cm)	-	-	-	+	+	+
	60s (15cm)	-	-	-	+	+	+
	20s (30cm)	-	-	-	+	+	+
	30s (30cm)	-	-	-	+	+	+
P. mallei TDJ29 and TDJ29^{M}	Wild-type TDJ29 TDJ29^{M}	+	+	+	+	+	+
	30s (15cm)	+	+	+	+	+	+
	60s (15cm)	+	+	+	+	+	+
	20s (30cm)	+	+	+	+	+	+
	30s (30cm)	+	+	+	+	+	+
P. oleovorans TDJ34 and TDJ34^{M}	Wild-type TDJ34 TDJ34^{M}	+	+	+	+	+	+
	30s (15cm)	-	-	-	-	-	-
	60s (15cm)	-	-	-	-	-	-
	20s (30cm)	-	-	-	-	-	-
	30s (30cm)	-	-	-	-	-	-

- Indicates no diesel degradation, +: Indicates diesel degradation, ++ Indicates high diesel degradation ability.

and in the most UV irradiated bacteria (mutants) except for *P. stutzeri* TDJ20^{M} after 10s and 30s UV irradiation and *P. stutzeri* TDJ24^{M} after 60s UV irradiation; which have given zero activity; no color change has been shown.

Culture of bacterial isolates on TSA media has also shown morphological differences between some UV irradiated bacterial isolates compared to the wild-types at different times, this was observed in: *P. stutzeri* TDJ20^{M} (at 15 cm distance from UV lamps) at all times, by colony color change from beige white to beige, *in P. stutzeri* TDJ24^{M} (at 15 cm distance from UV lamps) after 30 and 45s, by color change from beige to beige brown; and after 60s, by colony color change from beige to beige green and in *P. mallei* TDJ29^{M} (at 15 cm distance from UV lamps) after 10, 20, 45 and 60s; by colony and media color from beige to brown. Results are summarized in Table (4).

Alkane Monooxygenase Activity: Alkane monooxygenase test was done before and after UV irradiation, on 10 bacterial isolates, which previously showed positive results for this test (*Pseudomonas aeruginosa* TDJ2, *Bacillus megaterium* TDJ3, *P. mallei* TDJ4, *Acinetobacter calcoaceticus* TDJ5, *Moraxella sp.* TDJ9, *Comanamas sp*. TDJ10, *Micrococcus roseus* TDJ19, *P. stutzeri* TDJ24, *P. mallei* TDJ29 and *P. oleovorans* TDJ34). As indicated in Table (5), UV irradiation has no effect on alkane monooxygenase activity in the following isolates: *Pseudomonas aeruginosa* TDJ2^{M}, *Micrococcus roseus* TDJ19^{M}, *P. stutzeri* TDJ24^{M} *and P. mallei* TDJ29^{M} compared to their wild-type isolates, indicated by the same yellow color intensity compared to the wild-type. While it has negative effect also on *Bacillus megaterium* TDJ3^{M}, *Acinetobacter calcoaceticus* TDJ5^{M}, *Moraxella sp.* TDJ9^{M} and *P. oleovorans* TDJ34 ^{M}alkane monooxygenase activity indicated by the persistence of blue color even after 24h incubation. UV irradiation has a positive effect on *P. mallei* TDJ4 (to yield the UV irradiated counterpart *P. mallei* TDJ4^{M}) alkane monooxygenase activity, after being irradiated for 20s and 30s at 30 cm distance of UV lamp from plate's cover, shown by more intense yellow color comparing to the wild-type, which indicates higher alkane monooxygenase activity.

Molecular Characterization

Genomic DNA Extractions from Wild-types and Their UV Irradiated Bacterial Isolates: Genomic DNA was extracted from each of the 13 bacterial isolates and their UV-mutants that showed the highest activity in C23O and alkane monooxygenase and they were as follow: *Pseudomonas aeruginosa* TDJ2 and TDJ2^{M} (30s), *Bacillus megaterium* TDJ3, *P. mallei* TDJ4 and TDJ4^{M} (20s and 30s), *Acinetobacter calcoaceticus* TDJ5, *P. putida* TDJ6 and TDJ6^{M} (30s), *Moraxella sp.* TDJ9, *Comanamas sp*. TDJ10, *Micrococcus roseus* TDJ19, *P. stutzeri* TDJ20 and TDJ20^{M} (30s), *P. stutzeri* TDJ24 and TDJ24 ^{M} (30s), *P. mallei* TDJ29 and TDJ29 ^{M} (20s), *Bordetella sp*. TDJ31 and *P. oleovorans* TDJ34.

Table 6: RAPD banding pattern profiles using three random primers (OPA3, OPA9, OPA18) one at a time

Bacterial isolates	OPA3	OPA9	OPA18
P. aeruginosa Wild-type TDJ2	250 bp	400 bp	600 bp
	400 bp	600 bp	1000 bp
	850 bp		
UV irradiated counterpart TDJ2^{M}	250 bp	400 bp	600 bp
	400 bp	600 bp	700 bp
	850 p	700 bp	900 bp
P. mallei Wild-type TDJ4	230 bp	500 bp	230 bp
	260 bp		260 bp
	500 bp		500 bp
	850 bp		850 bp
UV irradiated counterpart TDJ4^{M}	230 bp	260 bp	500 bp
	850 bp	500 bp	230 bp
	260 bp	500 bp	850 bp
UV irradiated counterpart TDJ4$^{M'}$	230 bp	500 bp	230 bp
	260 bp		260 bp
	500 bp		500 bp
	850 bp		850 bp
P. putida Wild-type TDJ6	260 bp	500 bp	500 bp
	400 bp	600 bp	750 bp
	850 bp	700 bp	
UV irradiated counterpart TDJ6^{M}	260 bp	500 bp	600 bp
	400 bp	600 bp	750 bp
	600 bp	750 bp	
P. stutzeri Wild-type TDJ20	500 bp	850 bp	500 bp
	850 bp	600 bp	1000 bp
		700 bp	
		1200 bp	
UV irradiated counterpart TDJ20^{M}	500 bp	500 bp	600 bp
	850 bp	1200 bp	1000 bp
P. stutzeri Wild-type TDJ24	150 bp	500 bp	750 bp
	250 bp	1200 bp	
	500 bp		
	850 bp		
	1500 bp		
UV irradiated counterpart TDJ24^{M}	850 bp	500 bp	500 bp
		1200 mp	750 bp
P. mallei Wild-type TDJ29	900 bp	260 bp	1000 bp
	950 bp	700 bp	1500 bp
		1500 bp	
UV irradiated counterpart TDJ29^{M}	500 bp	500 bp	500 bp
	850 bp	600 bp	
		1000 bp	
		1200 bp	

Numbers in bold: Indicate polymorphic bands between the wild-type bacterial isolates and its UV irradiated mutant.

Fig. 1: Detection of difference in C23O gene, after UV irradiation, by PCR amplification of C23O gene fragment using DEG-F and DEG-R specific primers on genomic DNA Lane 2: *Pseudomonas aeruginosa* TDJ2^{M}, lane 3: *P. putida* TDJ6^{M}, lane 4: negative control (without DNA template) and lane 1: 1kb DNA ladder. Electrophoresis was carried out in 1.5% agarose gel

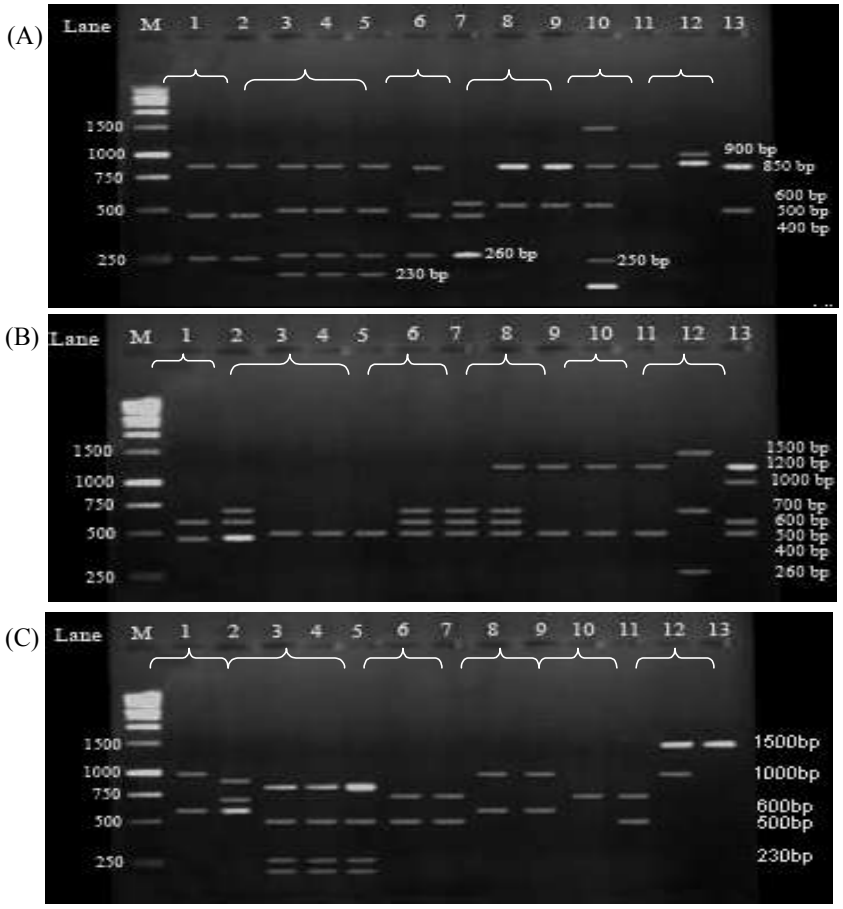

Fig. 2: Random Amplified Polymorphic DNA patterns generated of genomic DNA template of wild-type bacterial isolates and their UV irradiated counterparts, with 3 different primers (OPA3 (A), OPA9 (B) and OPA18 (C)). Lane1: *Pseudomonas aeruginosa* TDJ2, lane2: *Pseudomonas aeruginosa* TDJ2^{M}, lane3: *P. mallei* TDJ4, lane 4: *P. mallei* TDJ4^{M}, lane5: *P. mallei* TDJ4$^{M'}$, lane6: *P. putida* TDJ6, lane7: *P. putida* TDJ6^{M}, lane8: *P. stutzeri* TDJ20, lane9: *P. stutzeri* TDJ20^{M}, lane10: *P. stutzeri* TDJ24, lane11: *P. stutzeri* TDJ24^{M}, lane12: *P. mallei* TDJ29, lane13: *P. mallei* TDJ29^{M} and lane M: 1kb DNA ladder. Electrophoresis was carried out in 1.5% agarose gel. (Numbers between brace indicate bacterial isolate and its UV irradiated mutant).

PCR Amplification Using Specific Primers for Catechol 2, 3 Dioxygenase of UV Irradiated Bacterial Isolates: As shown before, that 30s UV irradiation of *Pseudomonas aeruginosa* TDJ2 and *P. putida* TDJ6 gave the highest level of catechol 2, 3 dioxygenase activities, PCR amplification using primer pair specific to this enzyme, was applied to the wild-type bacterial isolates DNA and their UV irradiated counterparts, to look for any difference in PCR product, there was no difference in PCR product size or number of bands (Fig. 1).

RAPD-PCR Analysis: RAPD analysis using three primers (OPA3, OPA9 and OPA18) generated different patterns were each primer, differences in amplified products were also revealed between wild-type isolates and their UV-mutant strains (Table 6).

Using OPA3 random primer, difference in RAPD PCR pattern was seen in *P. putida* TDJ6 (3 bands were revealed of 260, 400 and 850 bp (polymorphic band)) while its mutant counterpart TDJ6^{M} (3 bands of 260, 400 and 600 bp (polymorphic band) were observed). *P. stutzeri* TDJ24 (5 bands of 150, 250, 500, 850 and 1500 bp sizes) while its mutant counterpart TDJ24^{M} (1 band of 850 bp size). *P. mallei* TDJ29 (2 bands of 900 and 950 bp sizes) while its mutant counterpart TDJ29^{M} (2 bands also of different sizes: 500 and 850 bp sizes). The rest of bacterial isolates (*P. aeruginosa* TDJ2 and TDJ2^{M}, *P. mallei* TDJ4 and TDJ4^{M}, *P. stutzeri* TDJ20 and TDJ20^{M}) revealed no polymorphism.

Using OPA9 primer, the polymorphism in PCR products was seen in *P. aeruginosa* TDJ2 (2 bands of 400 and 600 bp sizes) while its mutant counterpart TDJ2^{M} (3 bands were revealed of 400, 600 and a polymorphic band of 700 bp sizes). *P. stutzeri* TDJ20 (4 bands of 500, 600, 700 and 1200 bp sizes) while its mutant counterpart TDJ20^{M} (2 bands of 500 and 1200 bp sizes). *P. mallei* TDJ29 (3 bands of 260, 700 and 1500 bp sizes) while its mutant counterpart TDJ29^{M} (4 bands of different sizes: 500, 600, 1000 and 1200 bp). The rest of bacterial isolates (*P. mallei* TDJ4 and TDJ4^{M}, *P. putida* TDJ6 and TDJ6^{M}, *P. stutzeri* TDJ24 and TDJ24^{M}) RAPD-PCR revealed no polymorphism.

Using OPA18 primer, the polymorphism in PCR products was seen in *P. aeruginosa* TDJ2 (2 bands of 600 and 1000 bp (polymorphic band) while its mutant counterpart TDJ2^{M} gave 2 polymorphic bands (700 and 900 bp) in addition to 600 bp band. *P. stutzeri* TDJ24 (1 band of 750 bp size) while its mutant counterpart TDJ24^{M} (2 bands of 500 and 750 bp). *P. mallei* TDJ29 (2 bands of 1000 and 1500 bp sizes) while its mutant counterpart TDJ29^{M} (one band of 1500 bp was observed) as shown in Fig.(2) and Table (6). The rest of bacterial isolates (*P. mallei* TDJ4 and TDJ4^{M}, *P. putida* TDJ6 and TDJ6^{M} and *P. stutzeri* TDJ20 and TDJ20^{M}) revealed no polymorphism.

DISCUSSION

Hydrocarbon contaminations have been the subject of continuous environmental and health concern over the last decades. The human health effects of hydrocarbons are well documented and exposure effects range from skin and lung irritation to cyanosis, to both human and wildlife [20]. The removal of these pollutants from the environment via natural physico-chemical and biological processes is, a slow and unpredictable way of counter-acting anthropogenic pollution and irreversible damage to the biosphere. Therefore, successful strategy to fight pollution is the use and manipulation of the detoxification abilities of living organisms particularly bacteria (bioremediation) [6]. Bioremediation strategies would be designed based on knowledge of the microorganisms present in the polluted environment, their metabolic abilities and how they respond to changes in environmental conditions such as high salinity.

The present study was done to create, by mutation (induced by UV irradiation), more effective oil degrading bacterial isolates, through increasing the activity of hydrocarbon-degradation key enzymes (Catechol 2, 3 dioxygenase and Alkane monooxygenase) and also testing their adaptability to salinity, so they could be applied in oil spilled brine soil and oil polluted sea water.

One of the important limiting factors for bacterial oil degradation is the adaptability to grow at high salt environments, which will not support externally added bacteria [21]. In the present study, all the thirteen oil degrading bacterial isolates were assayed for their tolerance to grow on different NaCl concentrations and also were assayed to grow on a sea-like water media (ONR7). The results, indicated that *P. mallei* TDJ4, *Moraxella sp.* TDJ9, *P. mallei* TDJ29 and *P. oleovorans* TDJ34 were able to grow on high salt concentrations up to (20% and 25%) and also on ONR7 medium. Those bacterial isolates could be consequently good candidates to be applied to clean high saline environments (sea water and soil) contaminated with hydrocarbons. The survival of the hydrocarbon degraders in the presence of high concentrations of NaCl implies that the bacterial isolates are adapted to extreme environments [21]. In the present study, no significant difference in salt tolerance was

detected in bacterial isolates before and after UV irradiation, this can be due to the low frequency of mutation to occur at two different genes.

Salinity may have a positive impact on hydrocarbons-degradation ability. Kerr and Capone [22] reported that there is correlation between salinity and rates of mineralization of some hydrocarbons. Fathepure [21] indicated that there were many explorations and production sites throughout the world that are contaminated with both oil and salt. This poses a problem for cleaning up those sites using bioremediation technologies since externally added bacteria will not support high salinity. On the other hand, though degradation of petroleum hydrocarbon compounds has been extensively studied, information on their degradation in oil-brine soil is scant. In addition, the presence of bacteria in brine soil has been discovered only recently; and it was shown that several types of hydrocarbon degrading microorganisms inhabit the brines and interfaces [23].

To improve the biodegradation ability of the thirteen bacterial isolates, random mutation using UV irradiation at two different intensities was applied; to select UV irradiated mutant strains with higher enzyme activity for oil biodegradation. Knowing that UV light is one of nonionizing radiation that causes the formation of crosslinked thymine dimmers [24]. C23Os are key enzymes in the catabolism of aromatics; knowledge of their catalytic mechanisms should give clues to the design of mutants with wider specificity and highest activity [25].

In the current study and in order to improve oil biodegradation ability of bacterial isolates, random mutation using UV irradiation was done on (*P. aeruginosa* TDJ2, *P. putida* TDJ6, *P. stutzeri* TDJ20, *P. stutzeri* TDJ24 and *P. mallei* TDJ29). According to the enzyme activity of C23O measured from different UV irradiated bacterial isolates, *P. aeruginosa* TDJ2^{M} and *P. putida* TDJ6^{M} (30s UV irradiation) showed the highest C23O activity by an increase of 30X and 20X times respectively, comparing to their wild-type isolates.

PCR using specific primers for C23O when done to (*P. aeruginosa* TDJ2^{M} and *P. putida* TDJ6^{M}) gave the highest C23O activity. No differences were observed in band pattern size or number. This may suggest that the mutation didn't occur within the complementary sequence for the two primers, but it may occur within the C23O gene sequence [26]. According to Meyer *et al*. [27], C23O activity in different species of the same genus could vary extremely. The wide variety of activities of C23O examined suggests heterogeneity in enzyme structure or gene regulation of the different isolates.

Few studies had analyzed the effect of single amino acid mutations on the functioning of C23O, for example the three active-site residues His199 (histidine), His246 and Tyr255 (tyrosine), which are strictly conserved in all known extradiol dioxygenases, may have important roles in the catalytic cycle and their mutation may modify or stop enzyme activity [28].

UV irradiation was also applied to the rest of bacterial isolates and their alkane monooxygenase activity was tested. As indicated in this study, monooxygenase pathway was monitored by examining and activating enzymes in bacteria using diesel as substrate, the reaction mixture became yellow due to the reduction of 2, 6-dichlorophenolindophenol (2, 6-DCPIP) which was blue in the oxidized form.

In the current study, diesel-degrading bacterial isolates (*P aeruginosa* TDJ2, *Bacillus megaterium* TDJ3, *P. mallei* TDJ4, *Acinetobacter calcoaceticus* TDJ5, *Moraxella sp* TDJ9, *Comamonas sp* TDJ10, *Micrococcus roseus* TDJ19, *P. stutzeri* TDJ24, *P. mallei* TDJ29 and *P. oleovorans* TDJ34) were subjected to UV irradiation to see their effects on monooxygenase activity. The UV irradiated bacterial isolate *P. Mallei* TDJ4^{M}, showed higher diesel degradation ability, thus higher monooxygenase activity compared with its wild-type counterpart. The absence of the activity of the two enzymes (C23O and monooxygenase) in some cases may be due to inflicted damage to the enzymes by UV irradiation causing reduced enzyme stability and shortened enzyme survival time [28].

To further test the effect of UV irradiation on the bacterial isolates, RAPD PCR was applied. Differences in RAPD-PCR patterns between wild-type and UV irradiated mutant bacterial isolates (*P. aeruginosa* TDJ2 and TDJ2^{M}, *P. mallei* TDJ4 and TDJ4^{M}, *P. putida* TDJ6 and TDJ6^{M}, *P. stutzeri* TDJ20 and TDJ20^{M}, *P. stutzeri* TDJ24 and TDJ24^{M} and *P. mallei* TDJ29 and TDJ29^{M}) were revealed. RAPD PCR detects differences along the entire bacterial genome, not only in particular sequences. Thus, this system is helpful in characterizing bacterial isolates over long periods and can find changes along the genome after UV irradiation of bacterial isolates [30]. Consequently, the use of RAPD-PCR can detect any differences between the genome of UV irradiated and non-irradiated bacterial isolates.

OPA random primers were used in different studies for the characterization of *Pseudomonas* species. One of those studies was done by Ortiz-Herrera *et al*. [30] for the characterization of *P. aeruginosa*. OPA9 has been vastly used for the characterization of *P. sp*., *P. putida* and *P. aeruginosa*.

In our study, OPA3 revealed differences in DNA banding patterns between the wild-type versus UV irradiated counterpart isolates in: *P putida* TDJ6 and TDJ6^{M} which gave a polymorphic band of 850 bp in wild-type and 600 bp in UV irradiated counterpart isolate and between wild-type *P. stutzeri* TDJ24 (5 bands: 150, 250, 500, 850 and 1500 bp) and its UV irradiated counterpart TDJ24^{M} (1 band: of 850 bp) and between wild-type *P. mallei* TDJ29 and its UV irradiated counterpart TDJ29^{M}, which gave 2 polymorphic bands in each.

OPA9 revealed polymorphism between the wild-type *P. aeruginosa* TDJ2 and its mutant TDJ2^{M} by an additional polymorphic band (700 bp) which was absent in the wild-type and between *P. stutzeri* TDJ20 and TDJ2^{M} revealed by 2 additional polymorphic bands (600 and 700 bp) in the wild-type and between *P. mallei* TDJ29 and TDJ29^{M} with different banding patterns.

The random primer OPA18 revealed polymorphism between the wild-type *P. aeruginosa* TDJ2 and TDJ2^{M} which gave polymorphic bands and between *P. stutzeri* TDJ24 and TDJ24^{M} which gave an additional polymorphic band of 500 bp in UV irradiated counterpart *P. stutzeri* TDJ24^{M} and between *P. mallei* TDJ29 and TDJ29^{M} which gave an additional polymorphic band of 1000 bp in wild-type *P. mallei* TDJ29.

This indicates that UV irradiation induced mutations in some wild-type isolates confirmed by shifting in banding patterns such mutation could be transition of C to T which cause formation of base pair TA instead of CG, which revealed larger or smaller size of DNA products, also caused by the creation or disappearance of primer sites and consequently cause new PCR products. As known, UV irradiation causes two cytosine or thymine residues to form a dimer. Subsequent DNA replication will produce CC to TT mutation; this could create or eliminate primer sites [24]. From the obtained results, the presence of adjacent pyrimidines in this primers: OPA3 (AGTCAGCCAC), OPA9 (GGGTAACGCC) and OPA18 (AGGTGACCGT), since UV irradiation has effects just the two adjacent pyrimidines (C or T) [28] and appearance of different fragment size may be due to the creation of other sites into genome sequence.

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