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Radiation resistant of native *Deinococcus* spp. isolated from the Lout desert of Iran "the hottest place on Earth"

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Abstract Two native ionizing radiation-resistant bacteria were isolated and identified from a soil sample collected from extreme conditions of the Lout desert in Iran. The hottest land surface temperature has been recorded in the Lout desert from 2004 to 2009. Also, it is categorized as a hyper arid place. Both ionizing radiation and desiccation may cause damage on genome. Soil sample was irradiated in order to eliminate sensitive bacteria then cultured in onetenth-strength tryptic soy broth medium. Bacterial suspension used for radiation treatment. Morphological and physiological characterization and phylogenetic studies based on 16S rRNA gene sequence were used for identification. The cells were rod shape, non-motile, non-spore forming and gram positive. The 16S rRNA gene sequence showed 99.5 % of similarity to Deinococcus ficus. Phylogenetic dendrogram demonstrated that the isolates branched with D. xibeiensis, D. ficus and D. mumbaiensis. Both isolates were resistant to >15 kGy of gamma radiation and $>600 \text{ Jm}^2$ of UV radiation. This is the first report on radiation resistant bacteria belonging to genus Deinococcus isolated from the Lout desert of Iran.

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Introduction

Members of genus Deinococcus are able to live in extreme conditions such as arid deserts, under ionizing radiations, ROS (reactive oxygen species) molecules and other oxidative stress inducing chemicals (Slade and Radman 2011). This astonishing ability in Deinococcus is due to its repair mechanisms (Minton 1994). It is well known that radiationresistant bacteria have evolved recombination repair and strong antioxidant systems to survive ROS-mediated damages and DSBs (double-strand breaks) (Daly et al. 2007). Moreover, pigments such as carotenoids and deinoxanthin, which is the major product of carotenoids pathway, play an important role in scavenging ROS and perform as an antioxidant (Lemee et al. 1997; Tian et al. 2007). Aim of this study is to investigate microorganisms that live in surface soil of desert where is exposed to high temperatures at days and low temperature at nights, also desiccation and rehydration. The Lout desert of Iran is known for its high surface temperature that is named as the hottest surface place on earth (Mildrexler et al. 2011). The Lout is the only place on earth that its surface temperature has been recorded more than 70 °C, measured by NASA satellite The Aqua/MODIS climate model grid (CMG). It had the highest surface temperature on earth between 2004 and 2009 in a range of 68.0-70.7 °C. (Mildrexler et al. 2011).

Many *Deinococcus* species has been isolated from deserts and arid places such as *D. deserti* that were isolated from the Sahara desert in Morocco (de Groot et al. 2005), *D. xinjiangensis* isolated from



Taklimakan desert of Xinjiang, China (Peng et al. 2009), D. gobiensis isolated from Gobi desert, Xinjiang, China (Yuan et al. 2009), D. peraridilitoris isolated from a coastal desert in Chile (Rainey et al. 2007), and D. navajonensis, D. hohokamensis D., apachensis, D. hopiensis, D. pimensis, D. maricopensis, D. yavapaiensis, D. sonorensis, D. papagonensis all isolated from soil collected from Sonoran Desert in Arizona, United states (Rainey et al. 2005). These bacteria possess efficient DNA repair mechanisms and so can survive (de Groot et al. 2005). Many of DNA damages such as double-strand breaks (DSBs) are caused by ionizing radiation which is the most lethal factor for organisms (Mattimore and Battista 1996). Deinococcus radiodurans is best studied member of this genus and is known as the most resistant bacteria of vegetative cells (Makarova et al. 2001). Some other microorganisms like Bacillus are also able to tolerate ionizing radiation by forming spores and protecting the genome with packaging it into nucleoprotein helices into a ring-like assembly in the absence of the water (Nicholson et al. 2000), but it is completely different from radiation resistance in vegetative cells like Deinococcus sp. Rubrobacter sp. Hymenobacter sp. and others that are able to tolerate radiation without forming spores (de Groot et al. 2005; Kim et al. 2008). A few numbers of strains isolated from non-irradiated samples demonstrate that the extreme radiation resistance of the organisms is not a result of selection of resistant strains by irradiation. This is a normal characteristic of these organisms, which is believed to be related to the desiccation resistance of these organisms (Mattimore and Battista 1996). D. radiodurans can withstand doses of radiation a thousand times higher than a human can. It survives under doses of radiation that do not exist naturally on earth (de Groot et al. 2005). It seems that this radiation resistance is related to the bacterial response to natural non-radioactive DNA-damaging conditions such as desiccation (Makarova et al. 2001). Many of Deinococcus members have been isolated from arid environments, i.e., desert soils or rocks (Hirsch et al. 2004; de Groot et al. 2005). We isolated two pinkpigmented strains with rod-shaped cells from soil samples of the Lout desert of Iran. These isolates were resistant to radiation. Morphological and physiological characteristics, radioresistant assay and phylogenetic analysis were performed. This is the first report of isolation and identification of *Deinococcus* in Iran.

Materials and methods

Sampling and isolation

Soil samples were collected from surface layer of sand in the Lout desert of Iran and stored at ambient temperature until transferred to the lab and processed. A primary treatment with gamma radiation was done to remove radiation-sensitive bacteria existing in the soil. Therefore, soil samples were exposed to gamma radiation, at a dose of 10 kGy (11.62 kGy h^{-1} ⁶⁰Co source; Gamma Cell 220), in Atomic Energy Organization of Iran (AEOI). After radiation, about 1 g of irradiated soil was transferred to tenfold diluted TSB medium (Merck) and incubated at 30 °C for 4 days (de Groot et al. 2005). Then grown bacteria were cultured on agar medium with the same nutrient as broth medium. Red- to pink-pigmented colonies were isolated on TGY Agar (1 % tryptone, 0.1 % glucose, 0.5 % yeast extract). All colonies were stored in TGY broth containing 15 % glycerol at -85 °C for further study.

Morphological and biochemical characteristics

For identification of the bacterial isolates, morphological and physiological characteristics were examined using the methods described in Bergey's manual of systematic bacteriology (Vos et al. 2011). Bacterial cell morphology and gram reaction was investigated using light microscope (Olympus CH40, Japan). Catalase activity was tested by adding a drop of 3 % hydrogen peroxide solution to the colony. Bubble formation indicates a positive reaction. Oxidase activity was determined by color change in oxidase test disks. Motility was determined by biochemical tests. The presence of spores was tested as explained by Ferreira (Ferreira et al. 1997). The temperature range of growth was determined on TGY agar incubated at 5-50 °C for 10 days. The pH range for growth was determined between 5.5 and 9.0 on agar plates at 30 °C as described by Ferreira (Ferreira et al. 1997). Assimilation of single carbon source tests was performed in phenol red broth base medium (Merck) containing carbohydrates.

Gamma radiation resistance

Samples were grown in TGY broth until reached exponential phase at OD₆₀₀ 0.5. Then washed twice by centrifugation and resuspended in 0.067 M potassium phosphate buffer at pH 7.0. Before using, the cultures were standardized at a final cell density of approximately 10^8 cfu m L^{-1} . The suspensions were divided into 5 mL aliquots and exposed to gamma radiation using a ⁶⁰Co source at AEOI with a dose rate of 11.62 kGy h⁻¹ on ice. After exposure to gamma radiation, suspensions were dilution plated in triplicate on TGY agar plates and incubated at 37 °C for 1 week. At the same time, Escherichia coli PTCC1330 served as a negative control. The cfu of each isolate were counted, relative survival was compared with unirradiated cultures, and survival curve was drawn.

UV radiation resistance

To determine the levels of resistance to UV radiation, isolates were grown in TGY until reached to a OD₆₀₀ of 0.2. Then harvested by centrifugation and resuspended in 0.067 M potassium phosphate buffer. About 100 µL of suspension were spared on TGY agar plates with their lids open and exposed to a 254 nm UV-LAMP at the dosages of 50, 100, 200, 400, 600 and 800 J m⁻². Light source was monitored using a J225 (UVP) radiometer to expose a dose of 4.5 J m⁻² s⁻¹ at a distance of 30 cm. *Escherichia coli* PTCC1330 served as a negative control. After exposure, plates were incubated at 30 °C for 1 week. Exposure was done in a dark room and irradiated plates were covered with aluminum foil to avoid photorepair process.

16S rRNA gene sequence determination and phylogenetic analysis

Genomic DNA was extracted using a standard bead beating method (Mohseni and Ebrahimi 2013). PCR amplification of the 16S rRNA gene was performed using universal bacterial primers PA and PH (Table 1) (Edwards et al. 1989). PCR amplification was performed in a MJ Mini thermal cycler (Bio-Rad) and cycling conditions described by Edwards et al. (1989). PCR product purified using GenJet purification kit (Thermo Scientific, Lithuania), then sequenced by GATC Company (Germany). The 16S rRNA gene sequences were analyzed against those available from

the National Centre for Biotechnology Information (NCBI) and EzTaxon databases using the BLAST search system to identify the most similar sequences alignment.

The 16S rRNA sequences were analyzed for anomalies using the Chromas Lite (2.01) software package, then assembled using the CAP contig program in Bioedit (7.1.3.0). FASTA and BLAST subroutines were used to determine closest relatives in the GenBank database. The sequences were compared with other closely related bacterial sequences from GenBank using the FASTA algorithm. Sequences were aligned and analyzed with the ClustalX program. Phylogeny reconstruction analysis was done by the maximum likelihood method with 1,000 bootstrap replicons in MEGA5 software. Tamura-Nei model was used to estimate the number of nucleotide substitutions per site between DNA sequences and evaluate evolutionary distances (Tamura et al. 2011).

Accession numbers of 16S rRNA gene sequence

The GenBank accession numbers for the 16S rRNA gene sequences of strains LD4 and LD5 are KF667511 and KJ154064, respectively.

Results and discussion

Isolation and identification of radiation resistant isolates

The Lout desert is known to be the hottest land surface on earth by the year of 2004-2007 and 2009 as reported by NASA satellite "aqua" (Mildrexler et al. 2011). It is also a very arid place and categorized as a dry desert (Mildrexler et al. 2011). Many members of Deinococcus genus were found in deserts; such as Deinococcus deserti (de Groot et al. 2005), Deinococcus peraridilitoris (Rainey et al. 2007), Deinococcus gobiensis (Yuan et al. 2009) and Deinococcus xinjiangensis (Peng et al. 2009). Deinococcus members have been found from harsh places such as radiation polluted places (Asker et al. 2009; Wang et al. 2010), atmosphere, stratosphere (Yang et al. 2010) and Antarctica (Hirsch et al. 2004). Environmental conditions in these places cause different kinds of damages to the DNA of organisms. Main relation between resistance to radiation and arid places is the same effect of them on organism's genome. There are several similar repair path-



ways and factors that help organisms to repair damages made by radiation and desiccation (Rainey et al. 2005).

Two isolates with pink colony which had resistance to radiation and survived in 10 kGy were isolated from soil samples of the Lout desert. The isolates were designated with LD4 and LD5. Growth occurred at 25–40 °C on TGY agar, but optimum growth temperature was 35 °C for both isolates. The isolated bacteria had a pH range of growth between 5 and 8 and optimum pH of growth was 7.0 \pm 0.2. Isolated bacteria were pale-pink in a convex colony. Cell

morphology was rode shape for both isolates while *Deinococcus* members comprise spherical to rod-shaped cell (Rainey et al. 2005). *D. radiodurans* have a spherical shape and occurs in tetrad (Makarova et al. 2001). Tetrads are also common in *D. geothermalis* and *D. murray* (Rainey et al. 1997), but *D. indicus* have rod-shaped cells like *D. maricopensis* and *D. yavapaiensis* (Rainey et al. 2005). Also, gram reaction of these isolates was positive like many other *Deinococcus* but *D. indicus* is one of the rare gram negative species (Suresh et al. 2004). In addition,

Table 1 Details of 16S rRNA universal primers (Edwards et al. 1989)

Primers ^a	Sequence $(5' \rightarrow 3')$	GC (%)	Anneal. Tem. ^b	PCR products
PA-F	AGAGTTTGATCCTGGCTCAG	50	56.0	1,500 bp
PH-R	AAGGAGGTGATCCAGCCGCA	60		

^a F forward primer, R reverse primer

^b Anneal Tem annealing temperature

 Table 2
 Morphological and biochemical characteristics related to *Deinococcus* species. Data of *D. gobiensis*, *D. indicus* and *D. radiodurans* are collected from Rainey et al. 2005

Characteristic	LD4	LD5	D. ficus	D. gobiensis	D. indicus	D. radiodurans
Cell morphology	Rods	Rods	Rods	Spherical	Rods	Spherical
Pigmentation	Pale-pink	Pale-pink	Pale-pink	Red-pink	Red	Red
Growth temperature ^a	35	35	nd ^b	30	30	30–37
Gram stain	+	+	+	+	_	+
Catalase	-	_	nd	+	+	+
Cytochrome Oxidase	-	_	+	+	_	+
Motility	-	_	_	_	_	-
Citrate	-	_	nd	nd	_	nd
Indole	-	_	_	nd	_	nd
Utilization of						
Glucose	-	_	nd	+	+	+
Sucrose	+	+	nd	+	+	+
Arabinose	+	+	+	+	-	+
Galactose	+	+	nd	+	+	+
Fructose	-	-	nd	-	-	+
Mannose	-	_	nd	+	+	+
Ribose	+	+	nd	+	+	+
Maltose	+	+	+	+	_	+
Degradation of						
Starch	+	+	nd	+	+	+
Casein	+	+	nd	+	+	+
Gelatin	_	_	+	+	+	+

^a ∘C

^b No data are available



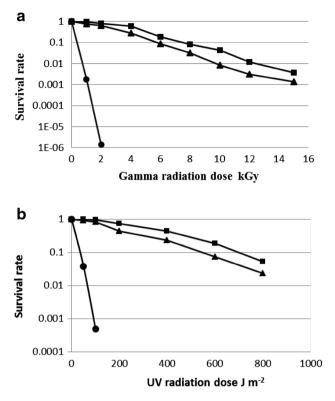


Fig. 1 Survival curve of radio resistant isolates after treatment with gamma radiation (**a**) and UV Light (**b**). LD4 (*filled square*), LD5 (*filled triangle*) and *E. coli* (*filled circle*)

these isolates are pale pink pigmented. Pigmentation is common in *Deinococcus* species. It has an antioxidant role in radiation-resistant microorganisms (Tian et al. 2007).

These isolates had a common biochemical characteristics with other members of *Deinococcus* genus like as being positive in gram reaction, hydrolysis of gelatin and starch and assimilation of carbohydrates and also had some different properties from other reported bacteria in this genus, such as being catalase and oxidase negative, being unable to hydrolyze gelatin unlike other members of genus *Deinococcus* (Table 2). Some biochemical characteristics showed interesting results. For example unlike many other strains, activity of catalase and oxidase activity was negative for these isolates. In addition, the isolates were able to utilize sucrose maltose and ribose but unable to utilize fructose, glucose, cellulose and mannose.

Radiation resistance

These strains were isolated from the surface soil of the Lout desert, which is exposed to an extreme cycle of desiccation and rehydration and also hot and cold weather conditions on day and night. So it could be expected to radiation resistant. Reduction in cfu of each sample under different doses of gamma radiation was monitored and the survival curve that had a sigmoid shape was drawn (Fig. 1). Numbers of radioresistant isolate LD4 were decreased from 7.5×10^7 to 2.68×10^5 cfu mL⁻¹ after treatment with 15 kGy of gamma radiation, and in LD5, it was reduced from 1.92×10^8 to 2.57×10^5 . D₁₀ value, the dose required to reduce the bacterial population to 90 %, was 7.15 kGy for LD4 and 5.94 for LD5 (Fig. 1a). These results demonstrated that isolated bacteria belong to highly radiation-resistant bacteria.

Radiation resistance is common in *Deinococcus* members, but a few species are also sensitive to ionizing radiation such as *D. radiomollis*, *D. claudionis*, *D. altitudinis* and *D. alpinitundrae*. These species were psychrophiles and found in alpine environments (Callegan et al. 2008). Survival curve of LD4 and LD5 under UV radiation were drawn. Results of Fig. 1b showed that the isolates are resistant to a dose of >600 J m⁻². Survival curves of the gamma radiation and UV light of *E. coli* PTCC1330 dropped sharply, while the two isolate LD4 and LD5 were extensively resistant to gamma and UV radiation.

Results of this study demonstrated that isolate LD4 is more resistant to gamma radiation than LD5. Probably, resistance to radiation depends on several gene products and other factors such as growth conditions (de Groot et al. 2005). Some genes related to radioresistance may have less expression or even be inactivated or absent in LD5 in comparison with LD4. This can reflect differences between isolates and is not a distinctive taxonomic feature. Resistance to radiation can be different in each strain of *Deinococcus* (Masters et al. 1991).

16S rRNA gene sequence and phylogenetic analysis

Gene sequence of the *16S rRNA* gene containing 1,409 and 853 nt was determined for isolates LD4 and LD5, respectively. Result of BLAST in EzTaxon database showed that the two isolate LD4 and LD5 share 99.5 % similarity to *D. ficus*. Phylogenetic dendrogram which was drawn based on *16S rRNA* sequence demonstrated that these isolates fell within *Deinococcus-Thermus* linage (Fig. 2). In addition, BLAST sequence similarity analysis and phylogenetic studies verified that isolates LD4 and LD5 belong to the genus *Deinococcus*. Species of the genus *Deinococcus* has been found to be in *Deinococcus-Thermus* lineage which



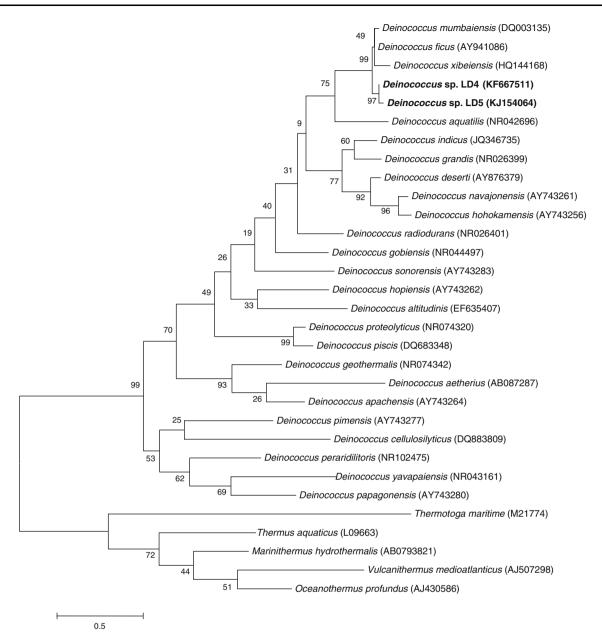


Fig. 2 Phylogenetic tree, based on *16S rRNA* gene sequences accessible from the NCBI database (accession numbers are given in parentheses), drawn after several alignment of the data by ClustalX. Clustering and distances were obtained using the software package

MEGA5 with the maximum likelihood method (Tamura et al. 2011). Bootstrap values based on 1,000 replications are listed as percentages at branching points. *Bar* 0.05 Knuc value

branches with family of Thermaceae to form a lineage in phylum level within the Bacteria domain (Hensel et al. 1986; Rainey et al. 1997 and Weisberg et al. 1989). Phylogenetic dendrogram demonstrated that *D. ficus*, *D. mumbaiensis*, LD4 and LD5 form a cluster that means have a high similarity. The *16S rRNA* gene sequence analysis showed that LD4 has a high similarity to *D. ficus* but it

showed some biochemical differences (Table 2). This study suggests that LD4 is a new isolate that belong to genus *Deinococcus*, but needs more evident to clarify that is it a novel species or not. DNA–DNA hybridization and chemotaxonomic studies such as fatty acid profile, respiratory quinine and G+C % of the genome are needed for a certain decision.

Conclusion

This is the first report of the radiation-resistant *Deinococcus* sp. isolated from the Lout desert in Iran. Two radiation-resistant bacteria were isolated and identified from extreme conditions of the Lout desert, known as a very arid and the hottest place in the world. These isolates were resistant to a dose of >15 kGy of gamma radiation and >600 J m⁻². Results of sequence analysis demonstrated that these isolates belong to *Deinococcus* genus and are closely related to *D. ficus*, but had some biochemical differences. Phylogenetic analysis showed the relationship between LD4 and LD5 isolates and other *Deinococcus* species. This study suggests that LD4 is a new but more studies need to show the novelty of isolates.

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