

Isolation of a Mesophilic and Halotolerant Strain of *Kocuria polaris* From Gandom Beryan Area in the Lut Desert of Iran, Moderately Resistant to Gamma Radiation and Desiccation

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Despite the extreme environmental conditions, hot arid deserts have a number of microhabitats that allow the evolution of unique extremophiles so as to being adapted to desiccation and ionizing radiation. There have been several attempts to demonstrate a link between radiation resistance and desiccation tolerance phenotypes. The diversity of ionizing radiation resistant bacteria was investigated in soil and surface sand samples collected from arid Gandom Beryan area located in the Lut desert in Iran, by exposing to different periods of dehydration in a desiccator. The surviving bacteria were recovered after plating on R2A, TSA and TGY agar media. After discarding the spore-forming isolates; Twelve orange, pink, yellow and white pigmented colonies forming Gram-positive cocci-shaped were obtained. The isolated strains on R2A agar were more diverse (9 isolates) compared with those on TSA (3 isolates) and TGY (without any isolates). A gamma radiation and desiccation resistant, coccoid Gram-positive, lemon-yellow to pale-orange pigmented actinobacterium, designated strain A10, was isolated from a mixture of sand samples. Morphological and Biochemical characteristics and phylogenetic analysis based on 16S rRNA gene sequences revealed that strain A10 belonged to the genus *Kocuria* and was closely related to *Kocuria polaris* CMS 76orT (99.4 % similarity). Strain A10 was shown to be resistant to gamma radiation up to 4 kGy and remained viable after desiccation for 28 days.

Key words: Mesophilic, Halotolerant, *Kocuria polaris*, Lut Desert, Iran.

Although all of the challenges are found in the extreme environments, a broad diversity of microorganisms was isolated from these habitats that are called “extremophiles”. Hot arid deserts with harsh environmental conditions such as low water availability, large temperature difference between day and night, high levels of solar

irradiance and lower amounts of nutrients are classified among extreme environments¹. Microorganisms, with high resistance to extreme desiccation and ionizing radiation which are two main factors for oxidative stress conditions, make up a group of extremophiles. Generating free radicals known as reactive oxygen species (ROS), these two environmental stresses are the major exogenous sources causing oxidative stress in a living cell. Oxidative stress is characterized by induced diverse damages to DNA as well as proteins and lipids. A number of cellular defense

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mechanisms to combat oxidative stress in desiccation tolerant and radiation resistant microorganisms include efficient DNA repair systems, pigments such as carotenoids, ROS scavenging enzymes (e.g. catalases, superoxide dismutases and peroxidases) and antioxidant complexes containing divalent manganese were been described².

There have been several studies regarding the possible correlation between microbial desiccation tolerance and radioresistance phenotypes^{3,4}. Due to the low environmental background gamma radiation doses on the earth, probably desiccation has been as a contributing factor to evolve extreme resistance against ionizing radiation during evolutionary process. The reason for this lies in the fact that the drylands were more common on the earth's surface, compared to environments with high ionizing radiation flux³. An illustration of this is the isolation of various radioresistant bacterial species such as *Deinococcus*, *Hymenobacter*, *Kocuria*, *Geodermatophilus*, *Kineococcus*⁵ and cyanobacterium *Chroococcidiopsis* from some arid deserts around the world⁶.

Based on the aridity index (AI), named as the ratio of precipitation to potential evapotranspiration, areas having AI less than 1 are considered as deserts which constitute over one-fifth of total land on the earth⁷. In the view of climate scientists, regions with average annual rains (AAR) of less than 400 mm per year are classified as dry lands or deserts⁸.

The Lut desert with an area of 199000 km², between 28° and 32° N latitude, is located in eastern part of Iran and is the 25th largest desert in the world. According to NASA's satellite temperature records, some parts of the Lut desert have experienced surface temperature more than 70°C between 2003 and 2010, which makes it a candidate for the title of "the hottest place on the earth". Gandom Beryan (translated as Toasted Wheat) hill extends an area of about 200 km² and is located in 80 km north of Shahdad in Kerman province, Iran (Figure 1). Surface and hillside of this area is covered in black volcanic lava with which a considerable amount of sun's energy is absorbed, leading to extraordinary increased temperature. Also, a saline river, locally called Shoor River, as the only permanent river, flows at

the heart of the Lut desert⁹. The Shoor river water has a total salt concentration of approximately 13.4% which is about 4 times that of normal seawater.

In order to understand microbial diversity of arid environments, many previous studies have been performed on the Atacama desert in northern Chile that were mainly conducted by NASA's astrobiology program as a Martian surface model on earth⁸. Little is known about the abundance and diversity of desiccation tolerant and radioresistant microorganisms in the Lut desert and to date, the only report is the isolation of two ionizing radiation strains of *Deinococcus* spp. from a soil sample collected from this unique habitat¹⁰.

Members of the genus *Kocuria* are non-endospore forming, non-motile, Gram-positive cocci which are classified within the family Micrococcaceae in the Actinomycetales order¹¹. At the time of the writing, the genus *Kocuria* comprises of 20 species (<http://www.bacterio.cict.fr/k/kocuria.html>) which have been isolated from a wide variety of environmental habitats such as air¹², rhizoplane¹³, desert soil⁵ and salt-fermented seafood¹⁴. Resistance to various oxidative stress-promoting agents including ionizing radiation, desiccation and UV-C radiation has been reported for the genus *Kocuria*^{15,16}. We isolated a new strain of *Kocuria* sp. from surface sand samples of Gandom Beryan hill in the Lut desert of Iran. This strain was shown resistance against desiccation and gamma radiation.

MATERIALS AND METHODS

Chemicals

All chemicals and culture media were obtained from Merck and Quelab Laboratories Inc. Genomic DNA extraction kit and PCR master mix were purchased from CinnaGen and Ampliqon, respectively.

Sampling and isolation methods

In this study, desiccation tolerance was used as the selective factor to isolate ionizing radiation resistant strains. Surface sand and soil were collected from Gandom Beryan hill in the Lut desert (31° 01' 00" N, 57° 40' 20" E) (Figure 1) and kept at the ambient temperature until processed. Five grams of each sample in 4 replicates were placed in a sterile glass petri dish and incubated

for 1, 2, 4 and 8 weeks in a desiccator containing silica gel at 30°C¹⁷. A petri dish was removed from desiccator at above mentioned intervals of time, appropriate dilutions prepared in sterile 0.85% NaCl solution (w/v) and plated on R2A, TSA and TGY agar media. After incubation at 30°C for 14 days, single colonies were purified on the same medium by successive cultivation.

16S rRNA gene sequencing

To extract DNA, strain A10 was grown on R2A agar at 30°C for 3 days, and then bacterial colonies transferred to one microtube. Genomic DNA was extracted using a DNP TM Kit (CinnaGen) according to the manufacture's protocol. The almost entire 16S rRNA gene was amplified with bacterial universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') as forward and 1492R (5'-GGTTACCTTGTTACGACTT-3') as reverse. PCR reaction was performed in a Reddy 2X PCR MasterMix (Ampliqon) using by a Thermocycler (Veriti 96-well Thermal Cycler, Applied Biosystems). The quality of PCR products were confirmed by electrophoresis on 1% agarose gel and then purified and sequenced by MacroGen services (Seoul, South Korea).

Phylogenetic analysis

Almost complete 16S rRNA gene sequence of the strain A10 (1400 bp) was compared to other published known sequences which are available in the GenBank database using BLASTn program (<http://www.ncbi.nlm.nih.gov>) and through EzTaxon server (<http://www.eztaxon.org>) and then aligned with the highest 16S rRNA gene sequences obtained from EzTaxon using ClustalX software¹⁸. A phylogenetic tree was constructed by the neighbor-joining method¹⁹ with bootstrap values based on 1000 replicates²⁰ contained in the MEGA software package, version 6.

Morphological and Biochemical characterization

Cell morphology was observed by using light microscopy with cultures grown on agar medium at 30°C. Gram staining was carried out by using the standard Gram reaction and was confirmed by using the KOH lysis test method²¹. Wet-mount method was used to examine motility. Colony morphology was observed on R2A agar and Antarctic bacterial medium (ABM; 0.5% peptone, 0.2% yeast extract and 1.5% agar; w/v) plates after incubation at 30°C for 48 h. All the

Biochemical tests were performed at 30°C. Catalase, oxidase, urease and gelatinase activities, production of indole, starch hydrolysis, methyl red and Voges-Proskauer tests were assessed as described by Holding and Collee²². Growth at different temperatures (4, 10, 20, 25, 30, 37°C), the pH range (pH 4-10, at intervals of 1.0 pH unit) and various NaCl concentrations (0, 1, 3, 5, 7, 10, 15 %; w/v) were performed on ABM agar plates. The pH was adjusted by using 5M NaOH or HCl. Single carbon source tests were carried out using minimal medium : 1.05% K₂HPO₄, 0.45% KH₂PO₄, 0.1% (NH₄)₂SO₄, 0.5% carbon source²³.

Desiccation tolerance assay

Strain A10 was grown in ABM broth medium at 30°C for 24 h. Cells were collected by centrifugation, then washed and suspended in 0.85% NaCl solution to achieve an approximate concentration of 107-108 cfu ml⁻¹. 25 µl aliquots were added to each well of standard 96-well microplate and dried inside a desiccator containing silica gel as a desiccating agent at 30°C. After 1, 2 and 4 weeks, a microplate was removed and 250 µl of sterile 0.85% NaCl solution was added to each well and pipetted to rehydrate the desiccated cells²⁴. The number of survivors for each desiccation time were enumerated by serial dilution and plating on ABM agar. Plates were incubated at 30°C for 5 days. The percentages of survival were evaluated using viable cells present in the original inocula. Tolerance to desiccation in strain A10 was compared to *Deinococcus radiodurans* R1 (ATCC 13939) and *E.coli* (PTCC 1329) as desiccation resistant and sensitive strains, respectively. *D.radiodurans* R1 was grown in TGY broth (1% tryptone, 0.1% glucose, 0.5% yeast extract) at 30°C and *E.coli* grown in Luria-Bertani medium at 37°C.

Gamma radiation resistance assay

A washed cell suspension was prepared as described above. The final cell suspension, in triplicate, was divided into 1 ml aliquots in sterile 1.5 ml Eppendorf tubes and irradiated at room temperature from a gamma cell 60Co source (Gamma Beam-150 Type B) at different doses (up to 6 kGy in steps of 1 kGy) of gamma radiation. An unexposed tube served as a non-irradiated control. After irradiation, the treated samples were serially diluted in sterile 0.85% NaCl, plated on solid ABM medium and incubated at 30 °C for 5 days to determine the number of colony-forming units (CFUs). *D.*

radiodurans R1 and *E.coli* were used as positive and negative controls, respectively. The survival ratios were calculated by dividing the surviving cells after irradiation to viable cells in the unexposed control.

RESULTS

Identification of the microorganism

The Lut desert of Iran contains a collection of unique geological phenomena and records of the world. Gandom Beryan, a black basaltic hill, is one of these. Also, NASA' aqua/MODIS satellite recorded one of the highest surface temperature on earth in this large desert⁹. During a study of the culturable diversity of desiccation tolerant and radiation resistant bacteria in the soil and sand dunes of Gandom Beryan area, a large number of colonies were obtained after different dehydration periods in a desiccator.

Isolates which were found by microscopic examination to be spore-forming species were discarded. A total of 12 pigmented colonies were recovered on R2A, TSA and TGY agar plates. Most of these isolates were obtained on R2A agar (9 isolates). R2A medium with a lower level of nutrients if is combined with longer incubation times can to be an excellent choice for recovering and stimulating the growth of oligotrophic bacteria found in desert soils.

A mucoid lemon-yellow pigmented colony on R2A agar medium, designated as A10, was isolated after 8 weeks incubation of a mixture sand sample in a desiccator. Colonies on ABM agar are smooth, round with an entire margin, translucent and pale-orange in color. Cells are non-motile, aerobic, non-spore forming, Gram-positive cocci, occurring in diads and tetrads. Strain A10 Grows between 4 and 37°C and Optimal growth occurs at 25-30°C on ABM agar. Also, it was able to grow in

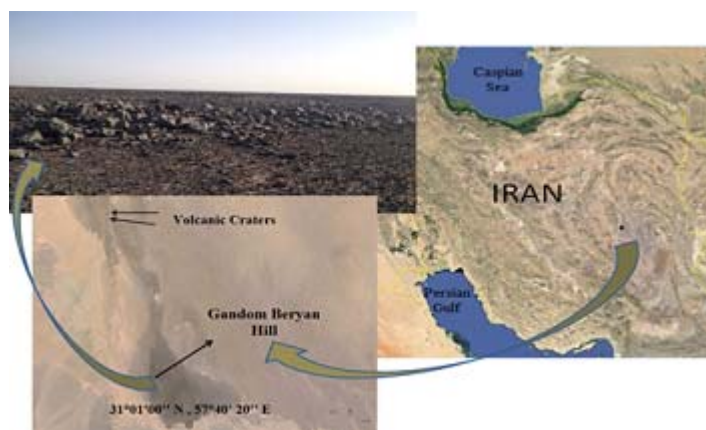


Fig. 1. The location of the Gandom Beryan hill and sampling site used in this study

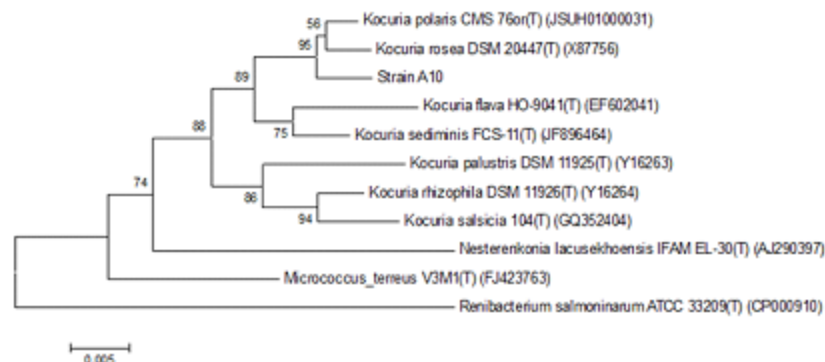


Fig. 2. Phylogenetic neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic relationship between strain A10 and other species of the genus *Kocuria*

the presence of 1-10% NaCl (w/v) with an optimal growth at 5% NaCl (w/v). ABM agar adjusted to pH 6–9 supports growth. The strain was positive for catalase, oxidase and starch hydrolysis but negative for indole, methyl red, Voges-Proskauer, urease and gelatinase. Lactose, D-maltose and L-arginine can be utilized as sole carbon sources for growth whereas L-arabinose and D-mannitol are not.

The almost complete 16S rRNA gene was sequenced. A search on the EzTaxon database showed that strain A10 belonged to the genus *Kocuria* and exhibited the most similarity with the 16S rRNA gene sequence from *Kocuria polaris* CMS 76 or T (99.4%) and *Kocuria rosea* DSM 20447T (99.2%). A phylogenetic dendrogram which was generated by the neighbor-joining method confirmed the taxonomic position of the strain A10. (Figure 2).

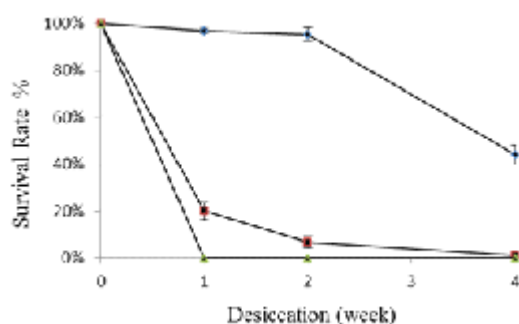


Fig. 3. Survival curves of *Deinococcus radiodurans* R1 (◆), strain A10 (■) and *Escherichia coli* (▲) exposed to desiccation stress

the viability of the *E. coli* reduced dramatically upon a week of desiccation, so that there were about 200 viable organisms per 100 μ l of original culture. The *E. coli* showed very little or no survival after 2 weeks of desiccation. Based on Figure 3, the *Kocuria* sp. A10 showed more resistant to desiccation than *E. coli* but has a lower tolerance to desiccation in comparison with *D. radiodurans* R1. It should be noted that as desiccator was opened at each time, the relative humidity within desiccator increased and after resealing, it decreased again. Therefore, the survival rates of bacteria may be affected by cycles of desiccation and partial dehydration³.

Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are given at nodes. Bar, 0.5% sequence variation.

Resistance to desiccation and gamma radiation

There is a close relation between desiccation tolerance and ionizing radiation resistance mechanisms³. Desiccation tolerance for the strain A10 after exposure to different periods of dehydration was investigated, as well as *D. radiodurans* R1 and *E. coli* strains were used for comparison. After 4 weeks in a desiccator containing silica gel, strain A10 remained viable and exhibiting approximately 0.7% survival, whereas the viability of *D. radiodurans* R1 decreased to about 54% over the same time period which was in agreement with previous studies^{16,24}. The survival percentages for strain A10 after 1 and 2 weeks were 18% and 6.6%, respectively. Also,

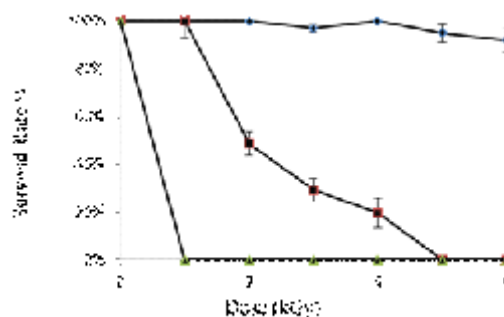


Fig. 4. Survival curves of *Deinococcus radiodurans* R1 (◆), strain A10 (■) and *Escherichia coli* (▲) following exposure to gamma radiation

Strain *Kocuria* sp. A10, *D. radiodurans* R1 and *E. coli* were tested for their resistance to different doses of gamma radiation. As shown in Figure 4, strain A10 could survive up to 4 kGy and showed no loss of viability at 1 kGy dose of gamma radiation. Strain A10 cells were moderately resistant to gamma radiation with survival rates of 49%, 29% and 20% at 2, 3 and 4 kGy doses of gamma radiation, respectively. *D. radiodurans* R1 culture showed highly radioresistance up to 6 kGy gamma rays dose without loss of survival. The cell numbers in *E. coli* culture was reduced dramatically after receiving a 1 kGy dose of gamma radiation.

DISCUSSION

According to the “desiccation adaptation hypothesis”, dehydration was a key selection pressure for the evolution of the extraordinary radioresistance in *Deinococcus radiodurans*, as one of the most radiation resistant organisms known. Indeed, ionizing radiation and prolonged desiccation have the similar effects on cellular macromolecules². Therefore, arid desert habitats with a low water activity is proposed as a logical candidate for the evolution of resistance against ionizing radiation.

Kocuria polaris was isolated by Reddy *et al* from a cyanobacterial mat sample in McMurdo Dry Vally, Antarctica. *Kocuria* spp. as a moderately radioresistance and desiccation tolerant bacteria has been reported previously¹⁶. Rainey *et al* isolated a *Kocuria* sp. from arid soil of Sonoran desert exposed to 5 to 9 kGy of gamma irradiation. In this study, a radioresistance strain of *Kocuria* sp., named A10, was isolated from sand dunes of arid Gandom Beryan area in the Lut desert by using desiccation conditions for the isolation of radioresistant bacteria. These results provided another substantial evidence of an evolutionary relation between ionizing radiation resistance and desiccation tolerance.

The Lut desert, the natural habitat of *Kocuria* sp. A10, is constantly exposed to some extreme environmental stresses including high levels of solar irradiance, starvation and dehydration. Strain A10 was 1800 times more resistant to desiccation than *E. coli*. However, *D. radiodurans* R1 showed higher resistance to 28 days of desiccation (65 times) when compared to strain A10.

Kocuria polaris A10 tolerates up to 10% NaCl and optimal growth occurs with 5% (w/v) NaCl. Based on these results, strain A10 has been adapted to a wide range of salt concentrations like other halotolerant species of the genus *Kocuria*²⁵. We hypothesized that the presence of a saline river in the vicinity of Gandom Beryan hill (not shown in Figure 1) can justify its high tolerance to salinity. Salt deposits is resulted from evaporation of the Shoor River's brine which can to be moved by strong winds in the Lut desert. Moreover, in contrast to its nearest phylogenetic neighbor, psychrophilic *K. polaris* CMS 76orT, strain A10

grows between 4 and 37 °C with an optimal growth at 25-30°C. Life in a hot desert such as the Lut desert with a high temperature difference between day and night requires such a temperature behavior.

Asgarani *et al* isolated a psychrotrophic *Kocuria* sp. ASB 107 from Ab-e-Siah radioactive spring in Ramsar, Mazandaran province, Iran. Radioactivity of Ab-e-Siah spring is approximately 146.5 Bq. l⁻¹. The equivalent dose around the spring is about 13.48 μSv.h⁻¹²⁶. The D10 value of ASB 107 strain (the radiation dose necessary to provide a 90% reduction in viability) for gamma rays was 2 kGy. Interestingly, radioresistance level of *Kocuria* sp. A10 at 2 kGy dose of gamma rays was 5 times higher than that of *Kocuria* sp. ASB 107. D10 value for *Kocuria* sp. A10 was about 4.5–5 kGy. These results can confirm the “desiccation adaptation hypothesis”. In other words, the Lut desert compared to Ab-e-Siah radioactive spring has been more suitable habitat for the evolution of high resistance against ionizing radiation.

It is well known that most radioresistant bacteria use two main strategy to achieve high radioresistance, including protection-based mechanisms by ROS-scavenging capacity such as Mn2+ complexes, carotenoids and antioxidant enzymes; and repair based mechanisms such as efficient well-developed DNA repair systems². Finding any relevant relation among the factors mentioned to radioresistance in *Kocuria* sp. A10 needs further study. In addition, challenging strain A10 by other sources of oxidative stress enhances better understanding regarding the mechanisms that confer resistance against oxidative stress.

Radiation resistant and desiccation tolerant bacteria have turned into an exciting topic in microbiology. Engineering radiation-resistant bacteria is an ideal candidate for bioremediation of radioactive waste sites²⁷. Many studies on biodiversity of extremophiles in the Atacama Desert, as a model of extraterrestrial life on Mars, have been performed by NASA⁸. Also, understanding radioprotective defense mechanisms in radiation resistant bacteria has opened not only a new promising approach for delaying aging, but also an alternative treatment for cancers². Gandom Beryan hill in the Lut desert of Iran has not been characterized from a microbiological standpoint until today. The study

of the microbial biodiversity of this area through both culture-dependent and -independent methods can help achieve the above aims in the future. In this study, Gandom Beryan hill in the Lut desert was selected for the isolation of radiation resistant bacteria. A mesophilic and halotolerant strain of *Kocuria polaris*, named A10, was isolated from a mixed sand sample collected from this arid area. Strain A10 was resistant at doses of 1 to 4 kGy of gamma radiation and maintained its viability over the period of 4 weeks of desiccation. A bioremediation strategy by using radioresistant microorganisms, such as *Kocuria* spp, can be developed to decontaminate the radioactive waste. Genetic engineering can be used to confer multiple resistance mechanisms against extreme environmental conditions in *Kocuria polaris* A10. Therefore, arid Gandom Beryan hill has been targeted by our research team for the isolation of radio resistant bacteria in long term.

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