



# Isolation and Characterization of Two Novel Radiation-Resistant Bacteria from a Radioactive Site in Iran

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## Abstract

Different groups of bacteria live in radioactive environments and microbial diversity of these regions depend on their geographical conditions. Overall, little is known about the bacterial diversity of naturally radioactive regions in Iran and the aim of this study was to investigate the biodiversity of radiation-resistant bacteria in a radioactive site in Ramsar, Iran. The soil samples were collected from the radioactive site and the samples were exposed to various doses of gamma radiation using a <sup>60</sup>Co source. After exposure, the samples were spread on TGY agar, and the surviving bacteria were purified and preserved for further studies. The 16S rRNA gene sequencing and the UV–Gamma radiation-resistant level of each strain were determined. After radiation and the cultivation of the soil samples, two Gamma and UV radiation-resistant, Gram-positive, yellow-pigmented, cocci, strictly aerobic, and catalase-positive bacterial strains were isolated. Phylogenetic analysis, based of 16S rRNA gene sequences, showed that the strains belonged to the *Kocuria* sp. and the *Dermacoccus* sp. In addition, both strains were resistant to > 15 kGy of gamma radiation and > 300 J m<sup>2</sup> UV radiation. An analysis of these strains constitutes the first report on radioresistant bacteria belonging to the *K. rhizophila* and *D. nishinomiyaensis* isolates recovered from the radioactive region in Iran. In addition, due to the radiation-resistant characteristic of our strains and their antioxidant activity properties, we supposed that these isolates could be ideal candidates for industrial and bioremediation applications.

**Keywords** *Dermacoccus* sp. · *Kocuria* sp. · Ionizing radiation · Ramsar

## 1 Introduction

Radiation constitutes energy in the form of particles or electromagnetic waves of Ultraviolet (UV) rays, X-rays, and Gamma rays. The most serious effect of radiation is oxidative stress and (Besaratnia et al. 2005) DNA breaking. Ionizing radiation and UV light are lethal to most bacteria, but ionizing radiation-resistant bacteria can tolerate the lethal effect of radiations; these bacteria have to be isolated from diverse environments such as dried food (Christensen and Kristensen 1981; Lewis 1973), sewage (Ito et al. 1983), paper mill machinery (Kolari et al. 2003; Vaisanen 1998), and irradiated

meat and fish (Grant and Patterson 1989). A number of radiation-resistant bacteria, including *Deinococcus*, *Acinetobacter*, *Chroococcidiopsis*, *Hymenobacter*, *Kineococcus*, and *Methylobacterium*, have been described. In addition, hyperthermophilic euryarchaeota species such as *Thermococcus* and *Pyrococcus* contain radiation-resistant strains; these strains are less resistant in comparison with *Deinococcus* and *Rubrobacter* (Rainey et al. 2005). Some other bacteria such as the *Bacillus* species are resistant to ionizing radiation through the formation of spores and their radiation-resistant mechanisms are different from other non-spore-forming radiation-resistant bacteria (Nicholson et al. 2000).

*Deinococcus radiodurans* is the most radiation-resistant bacterium and can survive in 1000 J m<sup>-2</sup> UV light and in more than 20 kGy of Gamma radiation—only 10 Gy of radiation kills most vertebrates (De Groot et al. 2005). Radiation is one of the most effective types of DNA-damaging agents, but radiation-resistant bacteria have several efficient systems for protection against radiation

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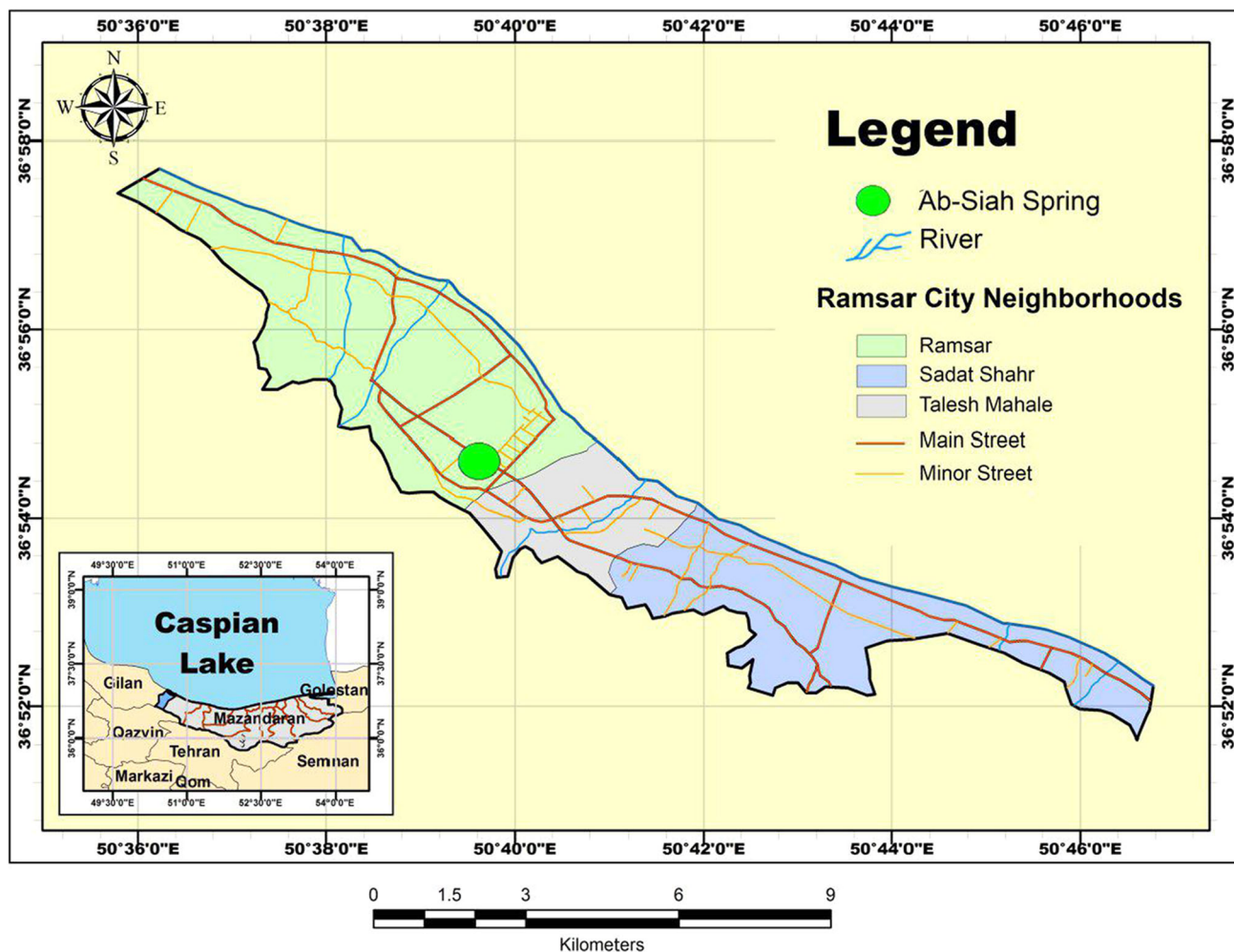


Fig. 1 The geographical location of Ramsar (Mazandaran, Iran, N36.49, E50.38)

and DNA repair (Slade and Radman 2011). In addition, substances included in pigments protect against radiation and oxidative stress (Asker et al. 2007). The study of this group of bacteria is not only important for the understanding the mechanism of resistance but also for their potential in the bioremediation of radioactive waste sites (Appukuttan et al. 2006). Generally, little is known about the bacterial diversity that exists in naturally radioactive regions and the origin of radiation resistance in prokaryotes living in such environments in Iran. The aim of present study was to isolate radiation-resistant bacteria from a radioactive site in the Mazandaran Province, Ramsar, Iran.

## 2 Materials and Methods

### 2.1 Sampling Area and Screening Procedure

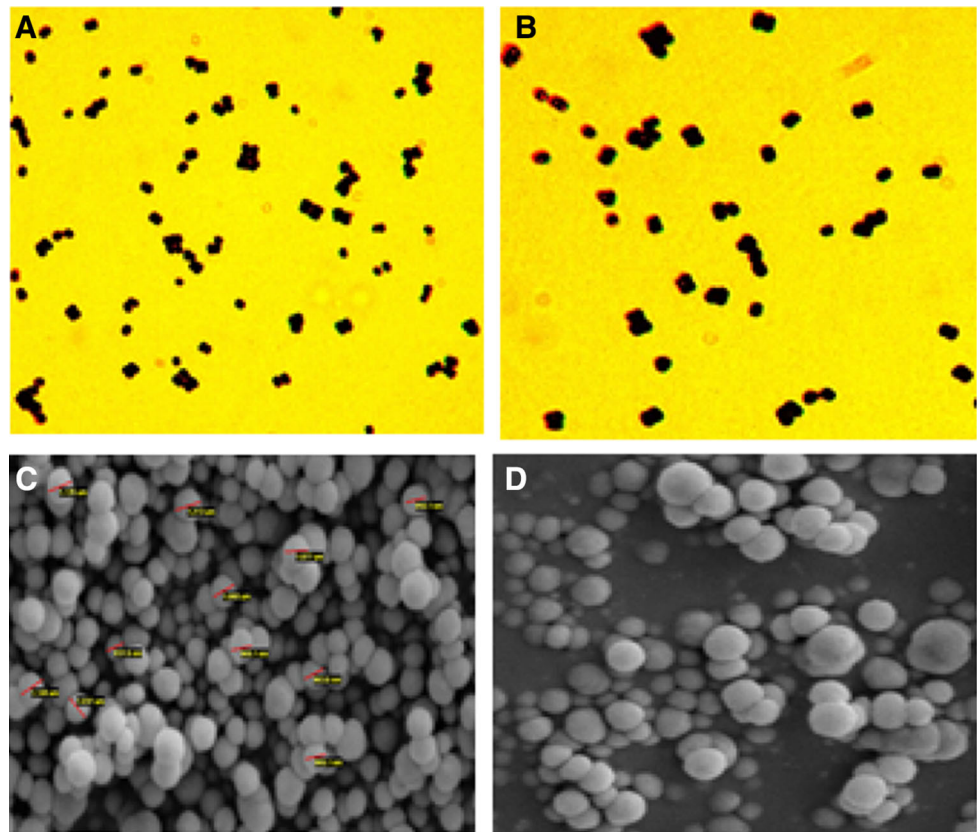
The study area was located in the southern part of the Mazandaran Province, Ramsar, Iran. Ramsar is known for

its high natural radioactivity, mainly due to the presence of  $^{226}\text{Ra}$ ,  $^{232}\text{Th}$ , and U ( $100\text{ mSv h}^{-1}$ ) (Fig. 1). For the isolation of radiation-resistant bacteria, 100 g of soil sample (upper 3 cm) was collected and the physicochemical properties of the soil sample were determined. One gram of the soil sample was irradiated using the  $^{60}\text{Co}$  source at a dose rate of  $7\text{ kGy h}^{-1}$  between 0 and 30 kGy. After radiation, serial dilutions of the samples were prepared and plated on the TGY agar (1% tryptone, 0.1% glucose, and 0.5% yeast extract) plates; these plates were incubated at  $30\text{ }^{\circ}\text{C}$  for 3–4 days. All the colonies were selected and preserved for further studies.

### 2.2 Gamma and UV Radiation Test

The Gamma radiation-resistance test for each strain was carried using cultures grown in TGY broth at  $30\text{ }^{\circ}\text{C}$  for 16 h. The cells were harvested at  $5000\times g$  for 5 min at  $4\text{ }^{\circ}\text{C}$ , and they were washed twice with physiological saline (0.85% NaCl). The cell pellet was suspended in 10 ml of

**Fig. 2** Morphology of F2 (a) and F3 isolates by light microscope (b) and scanning electron micrograph (c, d) indicating tetrad form. Phylogenetic tree showing the relationship between *Kocuria* sp. strain F2 (Accession Number: KY859854) and *Dermacoccus* sp. strain F3 (Accession Number: KY859853) isolates and other type strains. Bootstrap values based on 500 replications are given at the branching points



physiological saline. The cells suspensions were irradiated with a  $^{60}\text{Co}$  source at a dose rate of  $7 \text{ kGy h}^{-1}$ . After exposure to 5, 10, 15, 20, and 30 kGy, the cell suspensions were diluted and 200  $\mu\text{l}$  of each suspension was plated on TGY agar. The colonies were observed after incubation at  $30^\circ\text{C}$  for 48 h, and the number of CFU was determined. For UV-resistance assay, the isolate was grown to the late exponential phase in the TGY broth, washed with saline (0.85% NaCl), and the OD600 was adjusted to 0.6. The washed suspension (5 ml) was placed on a sterile Petri dish and irradiated using the UV source (Wilber Lourmat, France) at 254 nm at an intensity of  $10\text{--}500 \text{ J m}^{-2}$ . The irradiated suspension was plated onto the TGY agar plate, incubated at  $30^\circ\text{C}$ , and finally the colonies were enumerated after 3 days. In addition, *Escherichia coli* (PTCC 1533) was used as negative control.

### 2.3 Morphological, Biochemical, and Physiological Characterizations

For the examination of the morphological characteristics, the morphology of the isolates were studied using light and scanning electron microscopy (KYKY-EM3200, Japan), and the size of the bacterium was determined. The motility test was performed by the hanging drop and the SIM test. The temperature range for growth was determined on TGY agar at

a temperature between 5 and  $50^\circ\text{C}$ . In addition, the growth at different pH levels (3–10) and NaCl tolerance (1–7%) were assessed on the TGY broth. Biochemical tests were performed according to the method described by Murray. The antibiotic sensitivity of the isolates was tested by antibiotic disks on Muller–Hinton agar (Smibert and Krieg 1994).

### 3 16S rRNA Gene Amplification and Phylogenetic Analysis

Genomic DNA of each of the isolates was extracted using a bacterial DNA extraction kit (Bioneer, Korea) according to the manufacturer's instructions. Polymerase Chain Reaction (PCR) amplification of the 16S rRNA gene was performed by PCR using the universal bacteria primers 5'-AGAGTTT-GATCCTGGCTCAG-3' (positions 8–27 on the *E. coli* rrs gene) and 5'-GTTACCTTGTTACGACTT-3' (positions 1492–1509 on the *E. coli* rrs gene) (Smibert and Krieg 1994). Sequence of PCR products was performed using the ABI automated DNA sequencer and phylogenetic analysis was performed using the MEGA software (Version 4.0) (Asker et al. 2007). Finally, the phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987). For the GenBank/EMBL/DDBJ accession number, the 16S rRNA gene sequences were submitted to NCBI).

**Table 1** Biochemical properties of F2 and F3 isolates

Characteristics	F2	F3
Cell morphology	Coccus	Coccus
Cell size (µm)	~1	~1
Colony pigment	Yellow	Yellow
Motility	–	–
Temperature for growth range	20–40	20–40
pH opt.	7	7
	+	+
Catalase	+	+
NaCl tolerance (%)	4	4
Carbon utilization		
Fructose	+	+
Rhamnose	–	–
Trehalose	+	+
Lactose	+ (–)	+
Fructose	+	+
Mannose	+	+
Gelatinase	–	+
Starch hydrolysis	+	+
Antibiotic resistant		
Sulphamethoxazole	S	S
Chloramphenicol	S	S
Tetracycline	S	S
Streptomycin	S	S
Novobiocin	S	S
Erythromycin	S	S
Penicillin	R	R

S, sensitive; R, resistant; + (–), weak

### 3.1 Pigment Extraction and Its Analysis

The extraction of the pigments was performed by the dimethyl sulfoxide (DMSO) method. At first, the pigments were analyzed using spectrophotometer in the wavelength between 260 and 700 nm, and the LC–MS test was applied to determine the molecular weight of the pigment (Asker et al. 2007). The free radical scavenging activity of the extract pigment was measured by 1,1-diphenyl-2-picrylhydrazil (DPPH) (Tian et al. 2007), as follows:

$$\text{Antioxidant activity (\%)} = \left\{ 1 - \frac{A_{\text{sample}}}{A_{\text{control}}} \right\} \times 100$$

## 4 Results

After radiation and the cultivation of the soil samples, two yellow-colored isolates, designated as F2 and F3 strains, could withstand the gamma and UV irradiation. The isolates were Gram-positive, non-motile, non-sporulating, and

aerobic. The images from the light microscope and scanning electron microscopy revealed the morphology of the F2 and the F3 strains to be coccoid (Fig. 2a–c). The average length of these bacteria was 1.5 µm. The optimum growth temperature was 30 °C for both strains. Growth was observed between pH values of 6 and 9, with an optimum pH value of 7. The morphological and the biochemical characteristics that are related to the two strains are provided in Table 1.

The PCR amplification and sequencing of the 16S rRNA genes was performed and the amplified product size was about 1406 bp. Multiple sequence alignment was carried out with the 16S rDNA sequences of our strains with 20 other types of strains. The phylogenetic analysis based on the 16S rRNA gene sequence indicated that the strains are closely related to the *Kocuria* sp. (strain F2; Accession Number: KY859854) and *Dermaococcus* sp. (strain F3; Accession Number: KY859853), with similarity values of 99%. The phylogenetic analysis presented by a neighbor-joining tree in Fig. 3 shows the relationship of the two strains with other similar species.

According to the results, the *Kocuria* sp. strain F2 and the *Dermaococcus* sp. strain F3 were resistant to 20 and 30 kGy to gamma radiation, respectively (Fig. 4a). In addition, the UV radiation resistance of the *Kocuria* sp. strain F2 and the *Dermaococcus* sp. strain F3 were 400 and 500 J m<sup>-2</sup>, respectively (Fig. 4b).

In our study, the pigments of the strains were soluble in methanol and its absorbance spectrum was indicated by maximum peaks in between 474 and 479 nm. Based on the absorption data and the LC–MS system (ESI *m/z* = 597 and ~ 569), the major carotenoids produced by the *K. rhizophila* and *D. nishinomiyaensis* belonged to Astaxanthin and Zeaxanthin, respectively (Fig. 5).

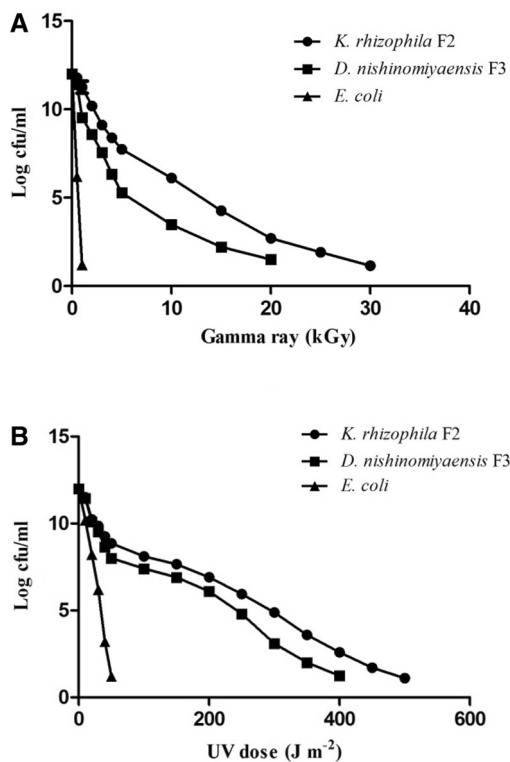
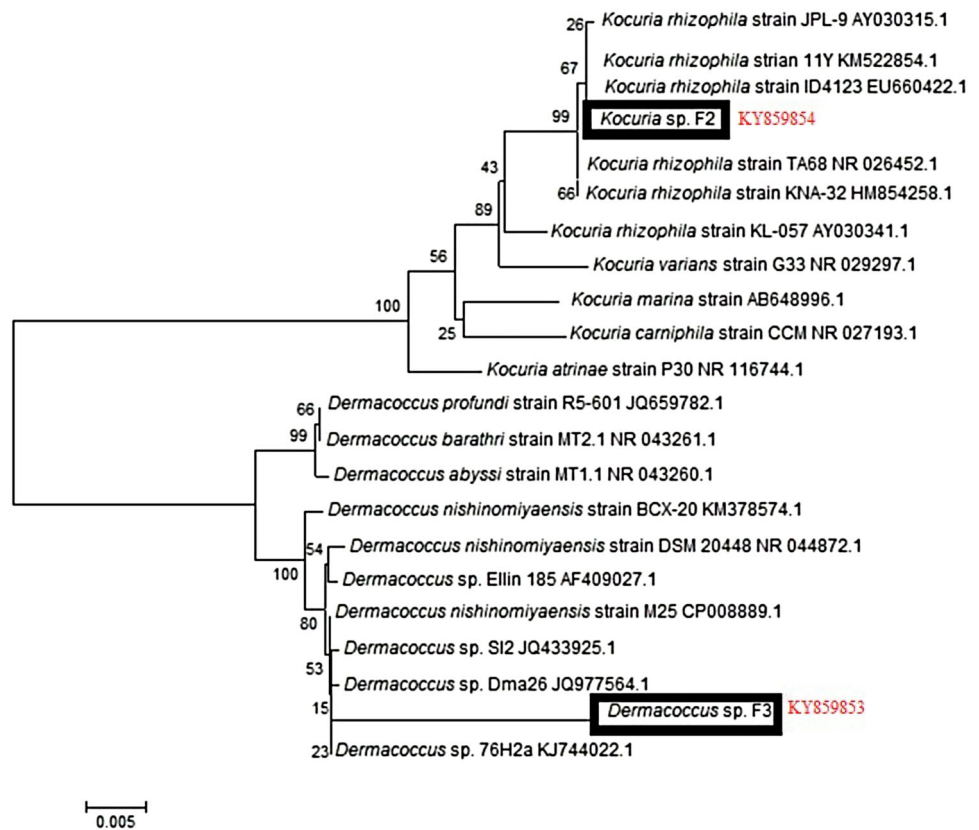
In addition, DPPH is a stable-free radical that is commonly used for the evaluation of antioxidant activity and the isolates (*Kocuria* sp. strain F2 and *Dermaococcus* sp. strain F3) showed DPPH-free radical scavenging activity of about 70 and 60%, respectively.

## 5 Discussion

The genus of *Kocuria* currently contains several species, but only a few studies have covered radiation resistance among the members of this genus. In a study by Brooks and Murray, two strains of gamma radiation-resistant *K. rosea* were characterized and taxonomically differentiated; one of these strains was formerly known as *M. roseus* UWO 157 and the other was called *M. roseus* (Stackebrandt et al. 1995). Strains of *Kocuria*, including *K. rosea*, have been isolated from various environments such as Misasa, a radioactive site in Tottori, Japan (Asker et al. 2007), a



**Fig. 3** Phylogenetic tree showing the relationship between *Kocuria* sp. strain F2 (Accession Number: KY859854) and *Dermacoccus* sp. strain F3 (Accession Number: KY859853) isolates and other type strains. Bootstrap values based on 500 replications are given at the branching points

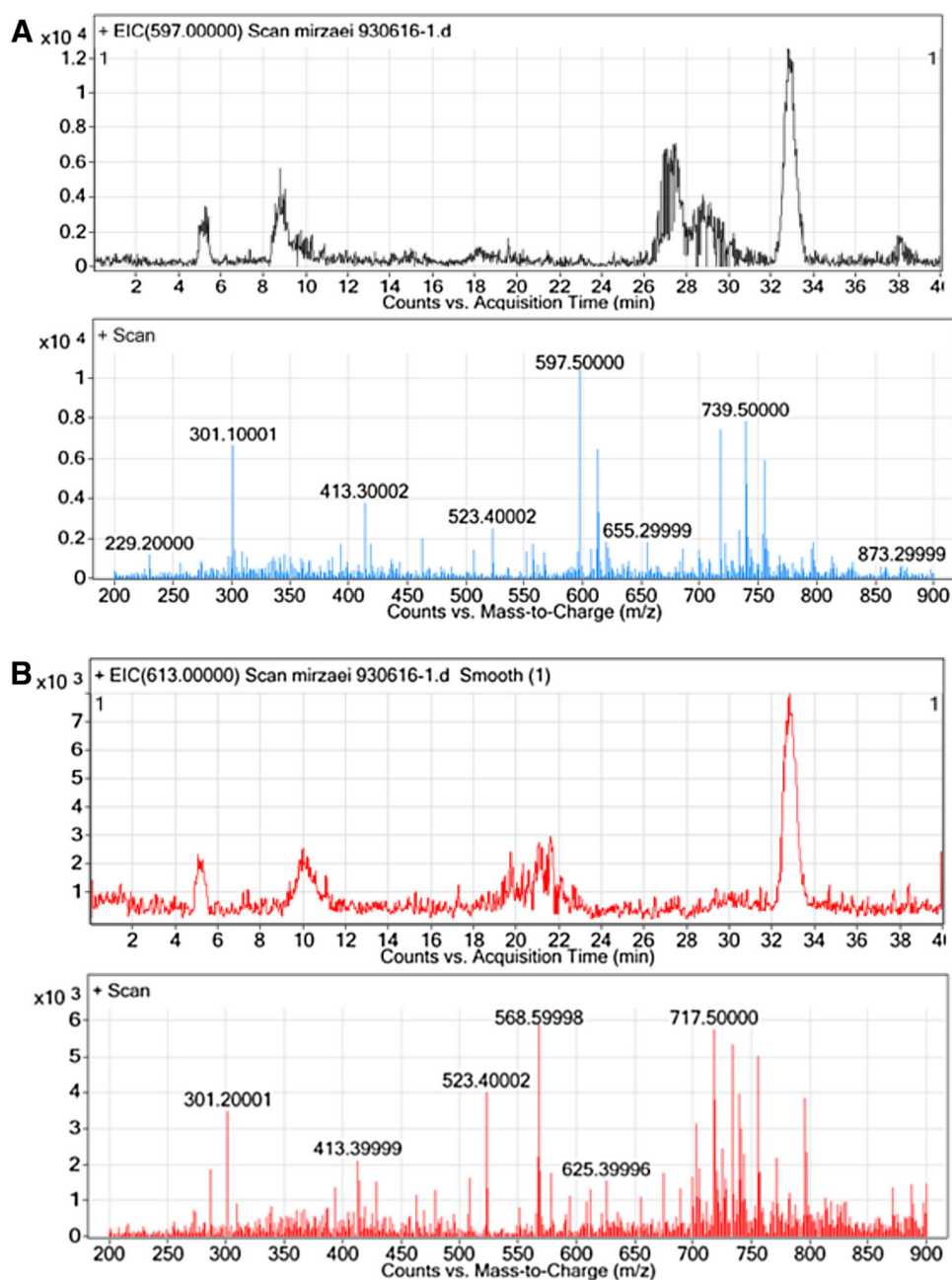


**Fig. 4** Survival curve of the isolates after gamma (a) and UV (b) radiation treatment. *Kocuria* sp. strain F2 (circle), *Dermacoccus* sp. strain F3 (square) and *E. coli* (triangle)

clean room (La Duc et al. 2007), a high-level nuclear waste-contaminated location at the Hanford Site in the State of Washington (Tian et al. 2009), the sand dunes of the Thar Desert, canned mushrooms (Shukla et al. 2007), and in the arid soil of the Sonoran Desert (Rainey et al. 2005). The genus *Dermacoccus* currently contains two species, *D. nishinomiyaensis*, a taxon that comprises strains initially classified as *Micrococcus nishinomiyaensis* (Kocur et al. 1975), and *D. abyssi* (Pathom-aree et al. 2006), which was isolated from the sediment collected from the Challenger Deep in the Mariana Trench. The environmental region used for bacterial isolation in this study should be classified as a stressful habitat. Using different doses of gamma radiation, radiosensitive bacteria were eliminated and radiation-resistant bacteria were isolated. Owing to high radioactivity, the total number of bacterial cells contained in this region is low.

Generally, the resistance of the strains to gamma radiation can be partly due to the presence of the enzymatic antioxidant defense systems including catalase, peroxidases, and non-enzymatic antioxidants (such as carotenoids), and molecular repair processes that function with high efficiency. Carotenoids are potential antioxidants and it was reported that carotenoids could have antioxidant and anticarcinogenic properties by scavenging from Reactive Oxygen Species (ROS) and acting as UV-energy

**Fig. 5** LC–MS analysis of the extracted pigment. Chromatogram shows a peak between at 32–33 min and ESI  $m/z = 597$  (a) and  $\sim 569$  (b) compared with the Astaxanthin and Zeaxanthin standard



absorbers. Shahmohammadi et al. (1998) suggested that the radioresistance of *Halobacterium salinarium* is partially due to carotenoids that act as effective in vitro antioxidants. It is known that *D. radiodurans* and *H. salinarium* mutants are defective in carotenoid production and they are more sensitive to ionizing radiation and hydrogen peroxide than their parental strains (Confalonieri and Sommer 2011). Astaxanthin and Zeaxanthin pigments are likely to protect these strains against UV light and gamma radiation. Overall, carotenoid pigments are one of the most widely used compounds in nutraceuticals, cosmetics, and the feed industries. The use of microorganisms in biotechnology to

produce carotenoids is approved by consumers and these are bioactive compounds in the pharmaceutical industries.

Radioactive sites are suitable sources for the isolation of pigmented bacteria and there are not many reports of the isolation of radioresistant and pigmented bacteria in Iran. In the present study, for the first time, we reported two radioresistant bacteria belonging to the *Kocuria* sp. strain F2 and the *Dermacoccus* sp. strain F3, which were capable of producing carotenoid pigments. It seems that these strains belong to the new isolates of the *Kocuria* and the *Dermacoccus* genera, but more tests including DNA–DNA hybridization and chemotaxonomic studies, such as fatty

acid profile, respiratory quinone, and GC % of the genome, are needed to confirm that these isolates are a novel species.

Isolation of novel ionizing radiation-resistant microorganisms from radioactive regions and/or other stresses is very important. The study of the molecular mechanisms involved in oxidative stress in microorganisms resistant to radiation show the evolutionary origin of prokaryotes, which in turn, find numerous applications. In addition, the isolated strains, F2 and F3, could be candidates to be engineered for use in bioremediation for the consumption of heavy metals in radioactive regions. Owing to antioxidant activities and pigment production, however, these strains can be useful for industrial and pharmaceutical applications, but additional experiments should be performed.

## 6 Conclusion

This study is the first report on the isolation of two new radiation-resistant bacteria, *Kocuria* sp. strain F2 and *Dermacoccus* sp. strain F3, which are isolates from Ramsar, a radioactive site in Iran. These isolates were resistant to 20 and 30 kGy to gamma radiation, respectively. According to the results of this study, it seems that these two isolates are new; more studies are needed to show the novelty of these isolates.

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## Compliance with ethical standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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