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Isolation of Radiation-Resistant Bacteria from Mars Analog Antarctic Dry Valleys by Preselection, and the Correlation between Radiation and Desiccation Resistance

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Abstract

Extreme radiation–resistant microorganisms can survive doses of ionizing radiation far greater than are present in the natural environment. Radiation resistance is believed to be an incidental adaptation to desiccation resistance, as both hazards cause similar cellular damage. Desert soils are, therefore, promising targets to prospect for new radiation-resistant strains. This is the first study to isolate radiation-resistant microbes by using gamma-ray exposure preselection from the extreme cold desert of the Antarctic Dry Valleys (a martian surface analogue). Halomonads, identified by 16S rRNA gene sequencing, were the most numerous survivors of the highest irradiation exposures. They were studied here for the first time for both their desiccation and irradiation survival characteristics. In addition, the association between desiccation and radiation resistance has not been investigated quantitatively before for a broad diversity of microorganisms. Thus, a meta-analysis of scientific literature was conducted to gather a larger data set. A strong correlation was found between desiccation and radiation resistance, indicating that an increase in the desiccation resistance of 5 days corresponds to an increase in the room-temperature irradiation survival of 1 kGy. Irradiation at −79°C (representative of average martian surface temperatures) increases the microbial radiation resistance 9-fold. Consequently, the survival of the cold-, desiccation-, and radiation-resistant organisms isolated here has implications for the potential habitability of dormant or cryopreserved life on Mars. Key Words: Extremophiles—*Halomonas* sp.—Antarctica—Mars—Ionizing radiation—Cosmic rays. Astrobiology 15, 1076–1090.

1. Introduction

Extremophiles are organisms surviving in environments considered to be physically or chemically extreme (Rothschild and Mancinelli, 2001). Extremophiles exhibit extraordinary tolerance to extremes of, for example, temperature, pH, or salinity. The exceptional resistance to ionizing radiation exhibited by the bacterium *Deinococcus radiodurans* was initially something of a puzzle, as it could tolerate exposures far higher than that ever encountered in the natural world. One proposed resolution was that *D. radiodurans* did not adapt to be radiation-resistant *per se* but has responded to selection pressures to improve desiccation survival (Mattimore and Battista, 1996).

Desiccation tolerance is the ability of cells to survive nearly complete dehydration through air drying (Billi and Potts, 2002). Dehydration causes severe disruption of enzymes and electron transport chains, which results in the accumulation of free radicals (Billi and Potts, 2002) and subsequently DNA damage (Dose *et al.,* 1992). Free-radical-mediated DNA damage also occurs under ionizing radiation exposure. Both desiccation and ionizing radiation cause a similar pattern of DNA damage and double-strand breaks. Therefore cellular mechanisms for recovery after desiccation are hypothesized to confer an incidental resistance to radiation (Mattimore and Battista, 1996; Billi *et al.,* 2000). Previous studies have found other organisms that exhibit resistance to both desiccation and radiation (Dose *et al.,* 1992; Billi *et al.,* 2000; Rainey *et al.,* 2005; La Duc *et al.,* 2007). For instance, Billi *et al.* (2000) found that strains of the cyanobacterial genus *Chroococcidiopsis* from both desert and hypersaline environments were also ionizing radiation–resistant. Similarly, Kottemann *et al.* (2005) reported that an archaeon,

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Halobacterium sp. strain NRC1, isolated from a hypersaline environment, was highly resistant to both desiccation and gamma irradiation. Sanders and Maxcy (1979) were able to isolate radiation-resistant species by selecting for desiccation tolerance, without exposure to irradiation. However, the nature of any correlation between desiccation resistance and radiation resistance has not been quantitatively investigated across a variety of different microorganisms, and is addressed in this study. Desiccation- and radiation-resistant multicellular organisms (*e.g.*, chironomids, tardigrades, rotifers, and nematodes) (Grewal *et al.,* 2002; Watanabe *et al.,* 2006; Jonsson, 2007; Gladyshev and Meselson, 2008) have different and more complicated cell repair systems, which is why they were not compared with microorganisms in this study.

Hypersaline or desiccating desert locations thus offer promising environments that favor the presence of incidentally radiation-resistant strains. The McMurdo Dry Valleys (MDV) in Antarctica are considered the coldest hyper-arid desert environment on Earth (Cowan *et al.,* 2014) and represent a terrestrial analog site for the martian surface. MDV have a total ice-free area of 4500 km^2, making them the largest (15%) ice-free land portion of the continent (Cary *et al.,* 2010; Levy, 2013). This region has a mean annual surface temperature of near −20°C (Doran *et al.,* 2002) with temperatures dropping down to −60°C in the winter (Horowitz *et al.,* 1972; Cary *et al.,* 2010). Frequent daily temperature fluctuations of >20°C often result in multiple freeze-thaw cycles (Aislabie *et al.,* 2006; Barrett *et al.,* 2008). Further environmental factors that pose extreme stresses on microbial life are the low bioavailability of water [<10 cm yr^{-1} water equivalent precipitation (Witherow *et al.,* 2006)], high MDV soil salt concentrations (Vishniac, 1993; Bockheim, 1997; Treonis *et al.,* 2000), steep geochemical gradients (Nkem *et al.,* 2006; Poage *et al.,* 2008), low levels of organic material in MDV soils (<1% by weight) (Vishniac, 1993; Burkins *et al.,* 2000), and elevated levels of ultraviolet B radiation (Smith *et al.,* 1992; Tosi *et al.,* 2005) caused by the ''Ozone Hole'' depletion of stratospheric ozone (Farman *et al.,* 1985; Jones and Shanklin, 1995).

The McMurdo Dry Valleys therefore offer a natural environment likely to contain desiccation-resistant and thus incidentally radiation-resistant microorganisms. In addition to simply culturing from soil samples, the successful isolation of radiation-resistant strains can be improved by high-dose radiation exposure before culturing, so as to preferentially select radiation-tolerant survivors. Such an approach has been previously attempted from different sampling locations. Ferreira *et al.* (1999) isolated novel thermophilic and radiation-resistant strains of *Rubrobacter* by gamma-irradiating samples from hot springs; Rainey *et al.* (2005) and Chanal *et al.* (2006) discovered new species of the *Deinococcus* genus by pre-irradiation of arid soil from the Sonoran and Tataouine deserts, respectively; Zhang *et al.* (2007) isolated a novel *Hymenobacter* species from irradiated Xinjiang desert sand; and Shukla *et al.* (2007) gamma-irradiated cell suspensions extracted from varied environments and isolated five ionizing radiation–resistant bacteria. This present study is the first to apply a preselection methodology to culturing from MDV soils.

A further motivation for studying MDV samples is in the interests of astrobiology. The habitability of the martian surface, with respect to terrestrial life, can be assessed by comparison to similar terrestrial environments and the extremophilic organisms found surviving in those conditions. Mars is believed to have had surface conditions in the past clement enough to allow for the development of life. The current environmental conditions on the surface would present a severe challenge to life, but the study of extremophilic microbes surviving in terrestrial habitats analogous to Mars suggests certain martian locales may remain habitable (Horneck, 2000). MDV soils are a meaningful analogue for the cold desert of the martian surface, in terms of very low temperatures, organic-poor soils, high levels of solar radiation, and desiccating conditions (Horowitz *et al.,* 1972; McKay, 1993; Wynn-Williams and Edwards, 2000). They have even been used as an analogue for the Mars Phoenix landing site (Tamppari *et al.,* 2012) and are considered to be a valuable research site for investigating microbial survival in the interests of astrobiology (Horowitz *et al.,* 1972; McKay, 1993; Wynn-Williams and Edwards, 2000).

One environmental hazard on the martian surface that is not reproduced on Earth is the ionizing radiation from the unshielded flux of cosmic rays (Pavlov *et al.,* 2002; Dartnell, 2011). Mars, today, lacks a substantial atmosphere or global dipolar magnetic field and so receives negligible protection from solar energetic protons accelerated by the sun or galactic cosmic rays originating from supernovae throughout the galaxy (Dartnell, 2011). The most energetic primary particles trigger extensive secondary cascades that penetrate through the top 2–3 m of the martian surface (Pavlov *et al.,* 2002; Dartnell *et al.,* 2007a, 2007b). Thus, ionizing radiation represents a hazard that penetrates much deeper than solar ultraviolet radiation and the oxidizing soil (Dartnell *et al.,* 2007a, 2007b). The Mars Science Laboratory Curiosity rover has measured an annual radiation dose of 76 mGy yr^{-1} on the martian surface (Hassler *et al.,* 2014). The low temperatures on Mars mean that any surviving microbial martian life, near the surface, is likely held dormant or cryopreserved. Periods of high obliquity on Mars are believed to produce high-enough temperatures and, thus, sufficient water activity in martian soils to permit episodic near-surface life reanimation and repair every tens of thousands to millions of years (Dartnell *et al.,* 2007b; Tamppari *et al.,* 2012). Consequently, the microorganisms would not be metabolically active and able to repair accumulating radiation doses for potentially thousands to millions of years. Resistance to ionizing radiation would, therefore, be another crucial cellular characteristic for the potential persistence of dormant martian microbial life accumulating radiation damage over time. In this way, the isolation of ionizing radiation–resistant bacteria from the cold, desiccated Mars analog MDV soils is also important for understanding the potential habitability of Mars.

Here, an irradiation preselection procedure was performed on soil samples from the cold desert environment of the Antarctic Dry Valleys. The aims of this study were twofold: to use irradiation preselection to attempt to isolate novel radiation-resistant bacteria from samples of MDV desiccated soils and to quantitatively investigate the nature of the correlation between desiccation and radiation resistance.

2. Materials and Methods

2.1. Antarctic Dry Valley sample collection

The environmental samples used in this study were collected from frozen MDV soils during the austral summer of February 2002 (Whiting, 2004). Sampling locations were

selected along a Miers Valley Transect (MVT), up the valley side from the floor, and so represent a diversity of altitudes and soil conditions. Sample MVT1 was taken at GPS coordinates 78°05.679′S, 163°48.271′E, ~169 m altitude, from wet ground in a floodplain. MVT4 was collected at 78°05.541′S, 163°48.310′E, ~183 m altitude, from dry, fine gravels at the base of the northern slope of the MVT. MVT12 was gathered approximately 15 m below the saddle of the Miers and Marshall Valleys, at ~820 m altitude, consisting of dry, fine gravels within a protected rock alcove. Samples were collected aseptically into sterile autoclaved polypropylene tubes (Nalgene), maintained at temperatures below 0°C throughout fieldwork and transit, before laboratory storage at −80°C (Whiting, 2004). Figure 1 displays the locations of these three sampling sites in the context of the MDV region.

2.2. Preselection for radiation resistance and strain isolation

Subsamples (3 g) of each of the MDV soil samples were placed in sterile universal tubes under sterile conditions, then maintained in storage at −80°C before irradiation. Gamma-ray exposure for the purposes of preselection for radiation-resistant microbes was provided by the colbalt-60 gamma-ray source at Cranfield University, Shrivenham, UK. Sample tubes were packed into dry ice in thin-walled polystyrene boxes to maintain a temperature of −79°C throughout the exposure, so as to suspend metabolic activity and emulate martian surface temperatures. Distance from the cylindrical source determined the radiation dose received, so samples were arranged in carefully determined circular arcs around the central source for the appropriate total dose. Further details on the radiation exposure setup are provided in Dartnell *et al.* (2010b). The Cranfield gamma-ray source is regularly calibrated with dosimetry, and an error of ±5% is estimated for the total doses delivered, including error in both the timing of the exposure and positioning of the sample (Dartnell *et al.,* 2010b). Triplicates of the samples were exposed to a total of 0, 1, 2, 4, or 6 kGy of gamma radiation, with the 0 kGy subsample from each dry valley site designated as the control. Control samples were exposed to no radiation from the source but were treated identically to the other samples in respect of the preparation, transport, and subsequent culturing procedure.

The aim of this study was to isolate novel radiation-resistant strains, so cells were cultured from the irradiated soils, starting with the samples exposed to the two highest doses of 4 and 6 kGy. Irradiated samples were thawed, 2 mL of sterile Dulbecco's phosphate buffer solution (PBS; Sigma)

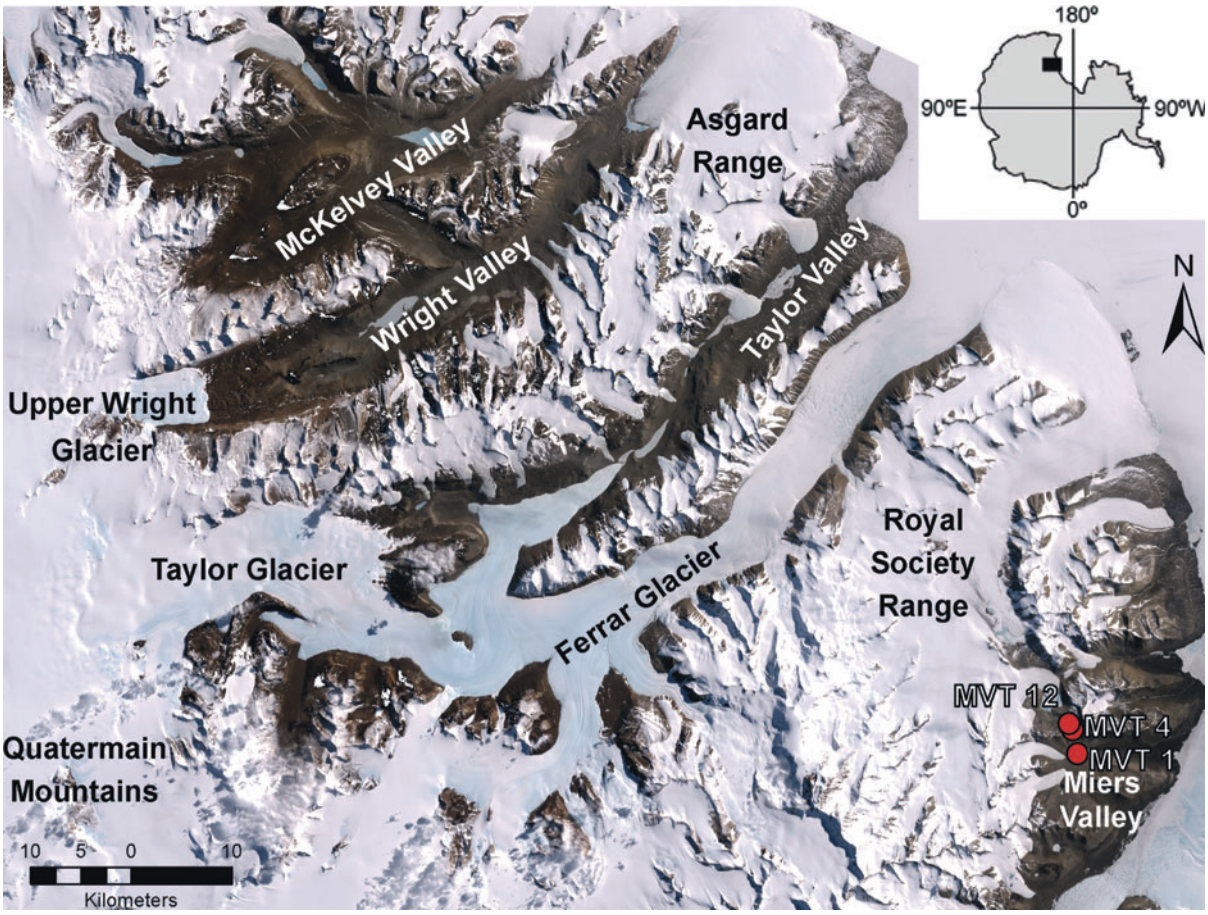

FIG. 1. Miers Valley Transect sampling locations indicated within the McMurdo Dry Valleys in Antarctica. The figure was prepared by the authors based on a NASA Landsat 7 photograph and ArcGIS using the GPS coordinates of the sampling locations from this study (Whiting, 2004). (Color graphics available at www.liebertonline.com/ast)

were added to each sample, and the sample tubes were vortexed for 1 min to dislodge cells into suspension in the fluid. Following a colony-forming unit (CFU) assay protocol, a 10-fold dilution series was performed on the suspensions over the range 10^{-1} to 10^{-5} cells g^{-1}. It was expected that MDV soil samples of this kind would contain approximately 10^4 cells g^{-1} (Gilichinsky, 2002; Dartnell *et al.,* 2010a, 2010b). From these dilutions, 50 μL were pipetted and spread on nutrient agar plates. Three formulations of nutrient agar were used for culturing: quarter-strength nutrient broth (¼ NB: Difco, 8.0 g per 1 L deionized water), quarter-strength R2A broth (¼ R2A: Difco, 18.2 g per 1 L of deionized water), and quarter-strength Czapek-Dox broth (¼ CZD: Difco, 35.0 g per 1 L of deionized water), each of which was solidified with 2% (w/v) agar (Difco). Quarter-strength concentrations of these standard microbiological culturing media have previously been found to be effective at culturing microorganisms from the MDV oligotrophic environment (Dartnell *et al.,* 2010b). The un-irradiated control samples were also processed as above to determine the initial viable soil population. In addition, a sterile plate (not spread with any sample diluent) of each media type was prepared to allow monitoring of possible contamination during the culturing procedure. Plates were incubated for 14 days at both room temperature (RT; 25°C) and 4°C to test for psychrophiles. Twenty-six colonies were sequentially subcultured by picking and replating to obtain pure isolates for sequencing. They were selected on the basis of their color, size, and colony morphology to capture as broad a range of phylogenetic and morphological diversity as possible.

2.3. Identification by 16S rRNA gene sequencing

The chosen isolates, which had survived the irradiation preselection, were identified to genus level by 16S rRNA gene sequencing. A single colony of each isolate was picked with a sterile pipette tip, resuspended in 50 μL of molecular-biology-grade water (molH_2O; Sigma), and vortexed. These suspensions were used as templates to amplify a 1.5 kb region of the 16S rRNA gene by polymerase chain reaction (PCR), using 27F (AGTTTGATCCTGGCTCAG) (Weisburg *et al.,* 1991) and un1492R (GGTACCTTGTTACGACTT) (DeLong, 1992) primers in a Techne TC-512 thermal cycler. PCR reactions were performed as described in Dartnell *et al.* (2010b). Following the manufacturer's instructions, the products were purified with a QIAQuick PCR Purification Kit (QIAGEN) then sequenced with the above primers. The returned forward and reverse sequences overlapped (600–800 bp of good gene sequence from each of the ends) and so allowed the reconstruction of the complete 16S rRNA gene (with sequences 1200–1400 bp long). These were used to identify the genus of each isolate through the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu), and the closest relatives were found by using the BLASTn (NCBI) sequence search utility (www.ncbi.nlm.nih.gov/BLAST). RDP was also used to construct a phylogenetic tree of the sequenced strains, which was displayed using the Interactive Tree of Life (iTOL; http://itol.embl.de), as shown in Fig. 3.

2.4. Determination of the radiation survival response

Four strains were selected for further characterization of their resistance to both desiccation and ionizing radiation. These four isolates were chosen because they belong to the genus *Halomonas*, which has not been studied previously in terms of ionizing radiation resistance. They will be referred to as Miers Valley Transect (MVT) 161, 463, 464, and 468 strains. Although originally isolated on quarter-strength media, they were found to grow faster on full-strength NB. They also grew equally well at RT as at 4°C; therefore the subsequent culturing was conducted at 25°C. The liquid cultures of these four strains were grown from the starter culture in 100 mL NB in 1000 mL conical flasks, incubated at 25°C for 4 days with constant agitation at 140 rpm. These stationary-phase liquid cultures were then washed of growth medium by centrifugation and resuspension of the cell pellet in PBS. The same cell suspension in PBS was used for both the following radiation and desiccation survival studies. Irradiation targets consisted of 1 mL subsamples in triplicate pipetted into sterilized 2 mL borosilicate clear glass vials (2-CV, Chromacol), stoppered with UV-sterilized 11 mm polyethylene snap caps (11-PEC1, Chromacol) and frozen at −80°C.

Samples were irradiated, at the Cranfield University facility again, and packed in dry ice to maintain a temperature of −79°C (as before, to suspend metabolic activity and emulate martian surface temperatures). They were arranged in circular arcs around the cylindrical cobalt-60 source to expose samples to a dose range of 1, 2, 4, 6, 8, 9, 12, and 15 kGy. Dartnell *et al.* (2010b) confirmed that cellular survival of irradiation is independent of dose rate at −79°C. Un-irradiated, but otherwise identically handled, samples were designated as controls.

After irradiation, samples were thawed to perform a serial dilution and CFU assay to determine the remaining number of viable cells. This CFU assay was performed in triplicate for each dose sample. The standard error of these independent cell counts was plotted as the data point error bar in population survival plots. Control (frozen but not exposed) samples gave the pre-irradiation population number. Therefore, the ratio of the irradiated sample viable cell count to that of the relevant control yielded the population survival fraction at each gamma-ray irradiation dose for each cell strain (Dartnell *et al.,* 2010b).

2.5. Determination of the desiccation survival response

The same cell suspension in PBS, as above, was used for the desiccation survival response experiment. For each selected strain, 100 μL subsamples of the liquid cultures in triplicate were pipetted into sterile 10 × 35 mm tissue culture dishes (Falcon). In addition to the four strains isolated in this present study, two other strains previously isolated from the Miers Valley and assessed for radiation survival, *Brevundimonas* sp. MV7 and *Rhodococcus* sp. MV10 (Dartnell *et al.,* 2010b), were also tested to characterize their desiccation survival. The tissue culture dishes were stacked in sterilized air-tight Nalgene tubs containing 100 g of granulated anhydrous calcium chloride as a desiccant ($CaCl_2$). A control dish with 100 μL of sterile NB was added to the tubs as a check for contamination. After set intervals, of between 1 and 42 days, dishes for each strain were removed from the tubes. They were rehydrated with 1 mL PBS and agitated for 15 min on an oscillating table at 70 oscillations/min to

recover the previously desiccated cells into suspension. Dilution series and CFU assays were then performed in triplicate on the recovered rehydrated samples to determine the remaining viable population (Dartnell *et al.,* 2010a).

2.6. Meta-analysis of published literature data

To gather a greater data set, a meta-analysis of previously published data in the literature was conducted. The strategy employed to find relevant papers combined key-word searches of online publication databases and subsequently checking those papers referenced by appropriate reports. To be suitable for inclusion, studies must have reported both desiccation and ionizing radiation survival experiments on the same microorganism. Those found were Romanovskaya *et al.* (2002), Kottemann *et al.* (2005), Rainey *et al.* (2005), Shukla *et al.* (2007), Callegan *et al.* (2008), and Slade and Radman (2011). This meta-dataset contains a total of 25 organism survival responses, although *Deinococcus radiodurans* was studied by multiple authors.

3. Results

3.1. Preselection for radiation resistance

Sampling locations along the MVT, within the MDV, are shown in Fig. 1. The environmental samples MVT1, MVT4, and MVT12 yielded culturable cells after 4 and 6 kGy of gamma irradiation. Cell counts for these three samples were averaged across all three growth media and both temperatures, since there were no significant differences in cell numbers between the nutrient and temperature conditions (Fig. 2). Figure 2 represents an approximate evaluation of the radiation resistance of the microbial community as a whole within the three MVT soil samples. For all samples, the cell numbers were reduced by over an order of magnitude by irradiation at 4 kGy and continued to decrease with increasing irradiation. No significant differences can be seen between the different sample types.

3.2. 16S rRNA gene sequence identification

Cells remaining viable from the soil microbial communities exposed to the two highest gamma-radiation doses, 4 and 6 kGy, were isolated in an effort to recover potentially radiation-resistant survivors. Each of the isolated radiation survivors was found to grow well at RT, rather than 4°C, thus expediting the subsequent desiccation and radiation resistance tests. All 26 strains were successfully sequenced. Figure 3 displays the phylogenetic tree calculated for the 26 sequenced isolates, as well as their closest relatives reported by BLASTn and related type strains. It was plotted using *Desulfurococcaceae* strain SRI-465 as the outgroup. The strains belong to three phyla: Firmicutes (*Staphylococcus* sp.), Proteobacteria (*Rhodobacter* sp., *Herbaspirillum* sp., and *Halomonas* sp.), and Bacteroidetes (*Hymenobacter* sp.).

The four isolates selected for further characterization of their radiation and desiccation survival response were strain 161 from MVT1 and strains 463, 464, and 468 (all three from MVT4). They were identified by 16S rRNA gene sequencing as species of the *Halomonas* genus (99% similarity based) (Fig. 3). They will be further referred to as *Halomonas* sp. MVT 161, *Halomonas* sp. MVT 463, *Halomonas* sp. MVT 464, and *Halomonas* sp. MVT 468. The reason these *Halomonas* strains were selected for further characterization of their radiation and desiccation survival

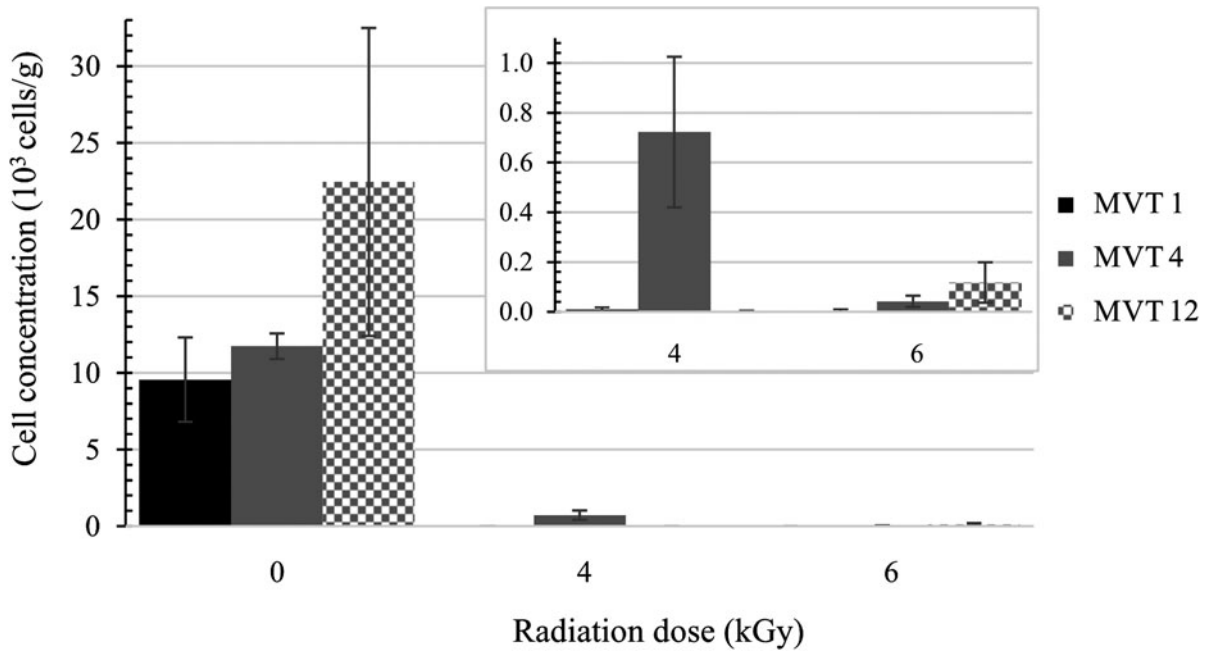

FIG. 2. Approximate MVT soil community radiation survival responses with cell counts averaged across all three nutrient media and both incubation temperatures. Error bars indicate the standard error of the population count replicates. Cell numbers were reduced by over an order of magnitude by irradiation at 4 kGy. No significant differences can be seen between the different sample types. Inset shows a rescaled view of the high-dose survival rates.

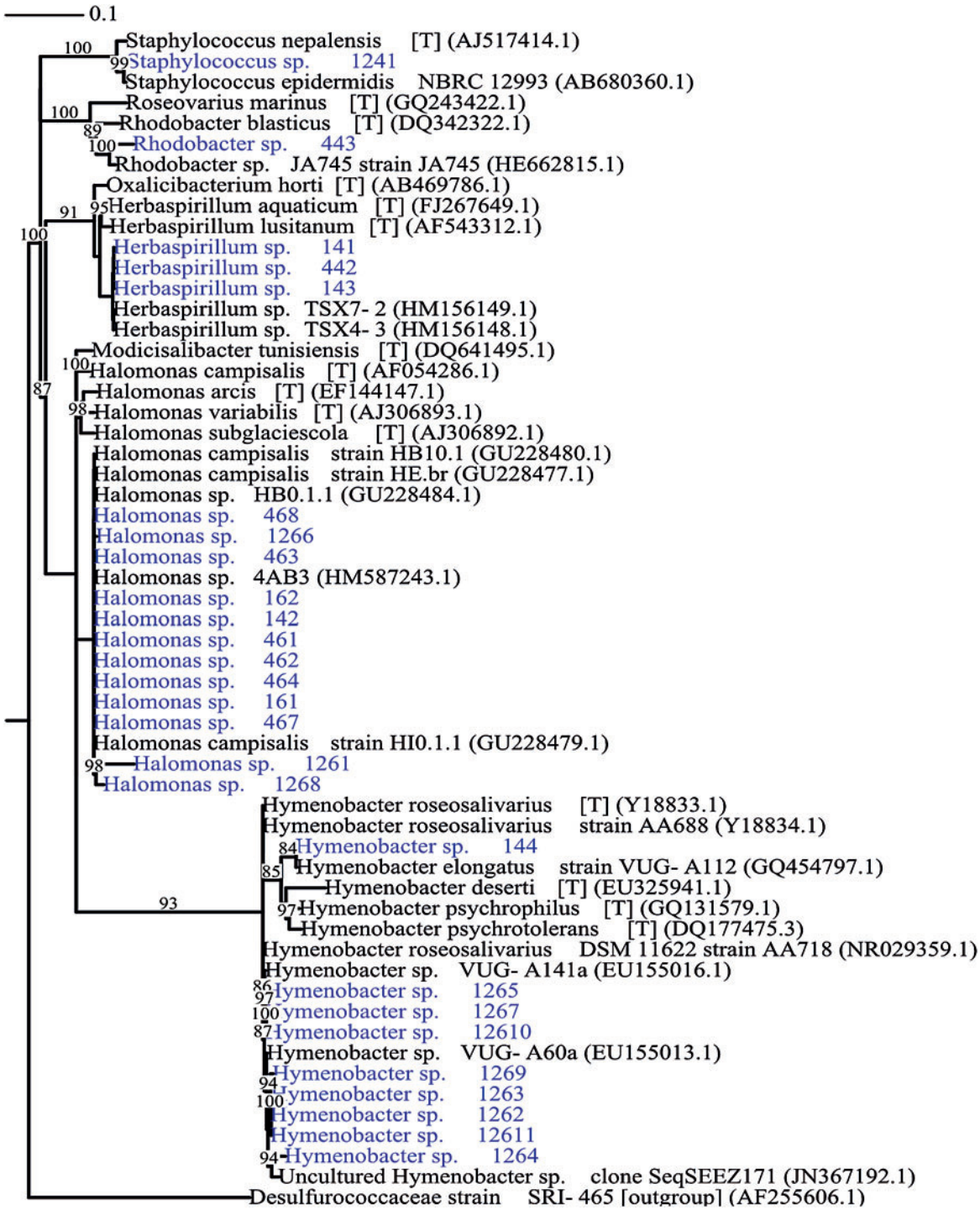

FIG. 3. Phylogenetic tree of the 26 bacterial strains isolated from MDV soil samples that had been irradiated to select for resistant survivors and identified by 16S rRNA gene sequencing (blue). The nearest relatives of the novel isolates found by BLASTn searches are also included (black). Most of the isolates were *Halomonas*, a halophilic genus, with *Staphylococcus* and *Rhodobacter* genera represented by a single isolate each. (Color graphics available at www.liebertonline.com/ast)

responses was that they are halophilic extremophiles, which have not been studied previously for their radiation resistance. Strains *Halomonas* sp. MVT 161 and 468 had bright yellow colonies with well-defined boundaries, whereas the strains *Halomonas* sp. MVT 463 and 464 had the same colony morphology but were light pink in color.

3.3. Radiation resistance

Figure 4 plots the surviving viable cell count of the four isolates *Halomonas* sp. MVT 161, 463, 464, and 468 after increasing doses of gamma radiation, normalized to the initial population. For comparison, the radiation survival

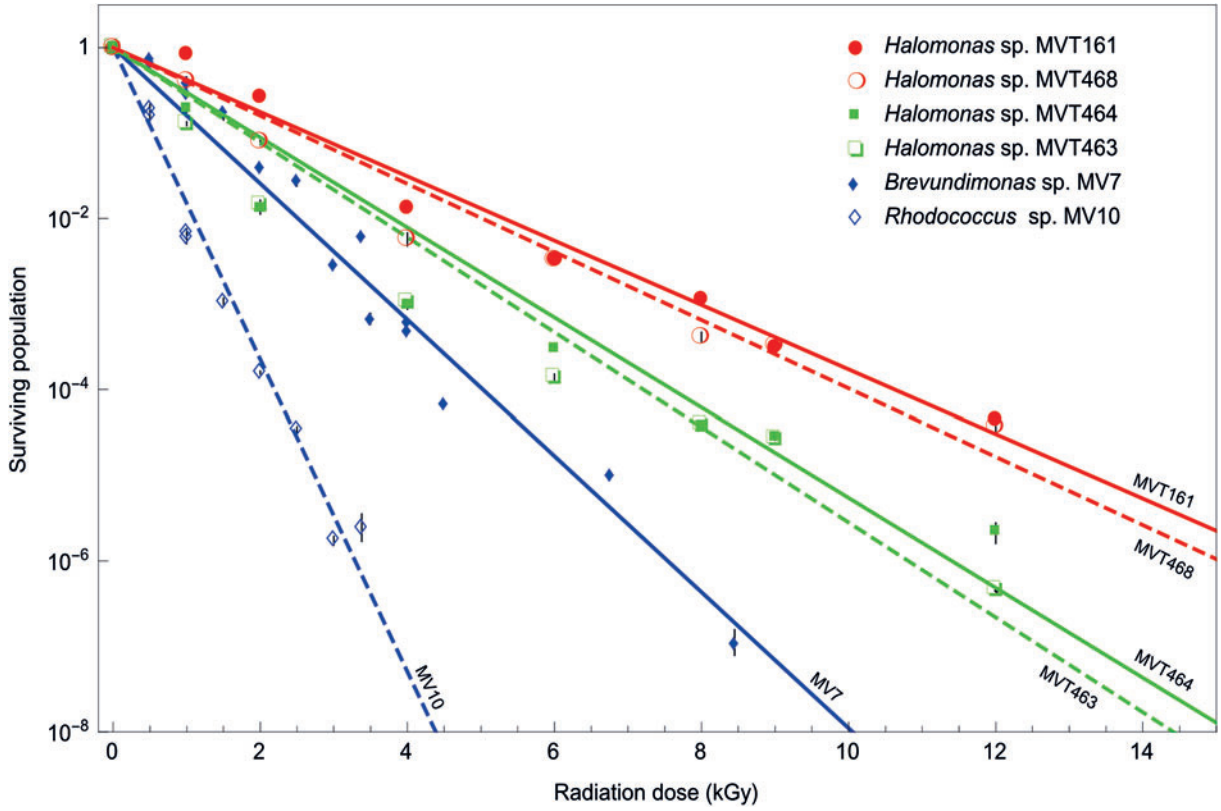

FIG. 4. Ionizing radiation survival plot of the four new *Halomonas* isolates. Exponential trend lines are fitted, and error bars indicate the standard error of the population count replicates. The survival responses determined previously (Dartnell *et al.,* 2010b) are shown for two Antarctic strains, *Brevundimonas* sp. MV7 and *Rhodococcus* sp. MV10. (Color graphics available at www.liebertonline.com/ast)

responses previously determined for two other Antarctic strains, *Brevundimonas* sp. MV7 and *Rhodococcus* sp. MV10, are plotted. They were also isolated from Miers Valley soils (Dartnell *et al.,* 2010b) but without an ionizing radiation preselection procedure. The survival response of all strains can be seen to follow a simple exponential decline (a linear trend in log-space) with radiation exposure. Consequently, trend lines are fitted to each data set with the form $y = e^{-a.x}$. All four *Halomonas* strains exhibit a radiation resistance significantly greater than the two strains previously isolated from Miers Valley soil without irradiation preselection (*Brevundimonas* sp. MV7 and *Rhodococcus* sp. MV10). *Halomonas* sp. MVT 161 exhibited the greatest radiation resistance of the strains tested here, although strain 468 was comparable. Despite being isolated from distinctly different micro-environments on the Miers Valley wall transect, these two strains also exhibit identical colony morphologies (bright yellow) and so could be representatives of the same species. Even though they show close 16S rRNA gene similarity with *Halomonas* sp. MVT 161 and 468, strains MVT 463 and 464 exhibit lower radiation resistance (though comparable to each other and with the same light pink colony appearance).

3.4. Desiccation resistance

The six strains tested for desiccation resistance were the four new Miers Valley isolates as well as the two previously isolated Miers Valley strains, *Brevundimonas* sp. MV7 and *Rhodococcus* sp. MV10 (Dartnell *et al.,* 2010b). Figure 5 plots the remaining population of viable cells, normalized to the initial cell count, as a function of the number of days desiccated. A more complex desiccation survival response is exhibited by all six strains than the simple exponential decay found with radiation exposure (Fig. 4). All strains show a rapid decline in the viable population over the first 5 days of desiccation. The gradient flattened in the continuing survival response up to the maximum exposure duration tested (42 days). Attempts were made to fit double exponential functions, of the form $y = e^{-a.x} + b.e^{-c.x}$, to these data. Nonetheless, they could not provide a satisfactory fit to the data series, so no trend lines are plotted in Fig. 5.

Survival of all four *Halomonas* isolates was reduced to a comparable surviving population of less than 1% after 42 days of desiccation. However, as shown in Fig. 5, strains 161 and 468 exhibit a similar response to each other and decline most readily at first before reaching the plateau. Conversely, strains 463 and 464 display a more steady decrease after the initial 2 days of desiccation. This pairing of responses repeats that seen in the radiation survival experiment. All four isolated strains show significantly greater desiccation resistance after 42 days than *Brevundimonas* sp. MV7 and *Rhodococcus* sp. MV10. The halomonads had an order of magnitude greater survival than the two strains cultured from Miers Valley soil without an ionizing radiation preselection (Dartnell *et al.,* 2010a).

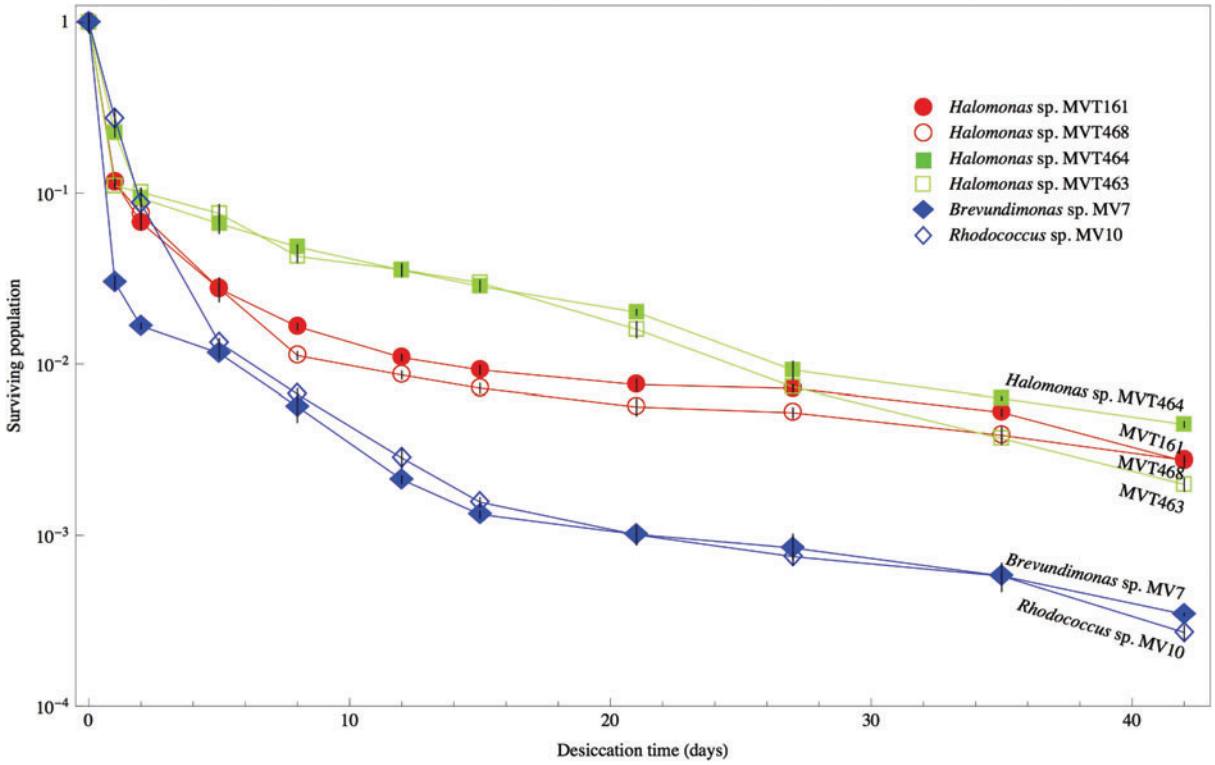

FIG. 5. Desiccation survival plots for the four new *Halomonas* isolates, along with those also determined here for the other MDV strains *Brevundimonas* sp. MV7 and *Rhodococcus* sp. MV10, isolated by Dartnell *et al.* (2010a). Error bars show the standard errors calculated for the population counts. (Color graphics available at www.liebertonline.com/ast)

The metric quantifying each organism's survival response to hazards such as desiccation and radiation is taken here as the D_{10} value. This is the level of exposure that results in 10% of the initial population remaining viable, which for desiccation survival is the number of days in the desiccator and for gamma-ray exposure is the total absorbed dose. D_{10} values were extracted from the ionizing radiation and desiccation survival responses (Table 1) for each of the four new *Halomonas* isolates, the other MDV strains *Brevundimonas* sp. MV7 and *Rhodococcus* sp. MV10 (tested here for desiccation resistance), and the radiation-resistant model organism *Deinococcus radiodurans* (Dartnell *et al.,* 2010b). These metrics of organism resistance gathered here are plotted against each other in Fig. 8, compared to data collected through literature meta-analysis.

3.5. Literature meta-analysis

Figure 6 displays the radiation survival data extracted from the literature meta-analysis studies. Pattern and color coding indicates the publication source (see figure caption). D_{10} values were extracted from 25 exposure experiments, where the population response line crosses the 10% survival threshold. Desiccation survival responses for each organism reported in these papers are shown in Fig. 7. Far fewer organism responses were reported as full survival plots. Results were given as either a D_{10} value (*e.g.*, points 21–24) or as the remaining viable population after 7 days (*e.g.,* point 9) and 42 days (*e.g.,* point 1) of desiccation.

Survival responses to both desiccation and radiation needed to be quantified in a similar way to test the correlation between the two environmental stresses. In the published literature, the reported desiccation resistance is the limiting factor, as full survival curves are often not provided and a range of different metrics are used in the community. Those studies were selected that either explicitly reported

TABLE 1. DESICCATION AND RADIATION SURVIVAL RESPONSES ARE SUMMARIZED WITH THE D_{10} METRIC

Organism	*Label*	*Desiccation D_{10} (days)*	*Radiation D_{10} (kGy)*
Halomonas sp. MVT 161	a	1.53	2.66
Halomonas sp. MVT 463	b	1.70	1.80
Halomonas sp. MVT 464	c	1.85	1.90
Halomonas sp. MVT 468	d	1.57	2.50
Brevundimonas sp. MV7	e	0.99	1.26
Rhodococcus sp. MV10	f	1.9	0.55
Deinococcus radiodurans	g	26	50

Data for the four *Halomonas* strains, *Brevundimonas* sp. MV7, and *Rhodococcus* sp. MV10 are extracted from Figs. 4 and 5. Desiccation data for *Deinococcus radiodurans* is taken from Dartnell *et al.* (2010a), using an identical methodology, and the radiation D_{10} value is extracted from Richmond *et al.* (1999), as this study extended to higher radiation doses at −79°C than Dartnell *et al.* (2010b). Letter labels correspond to those for data points in Fig. 8.

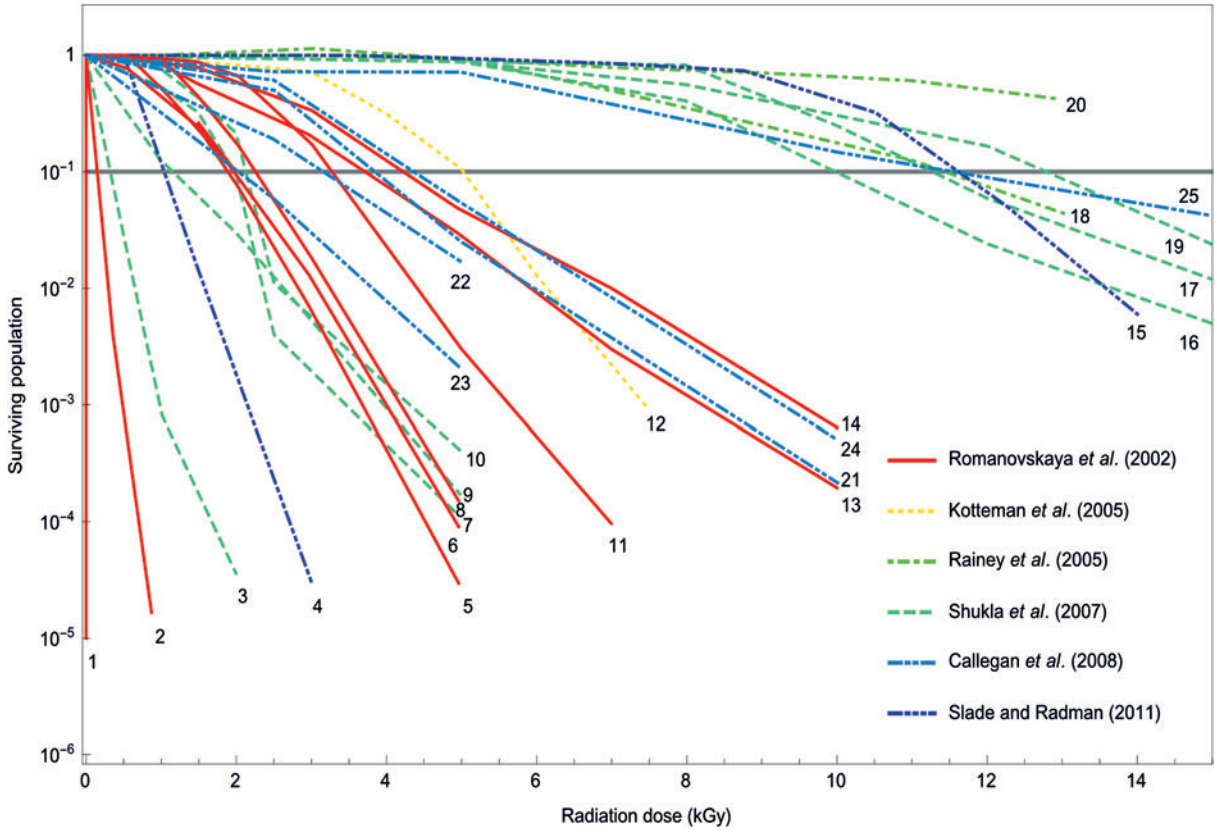

FIG. 6. Ionizing radiation survival data for varied microbes extracted from the literature meta-analysis. The D_{10} survival threshold is indicated with a thick horizontal line at 10% remaining population. Pattern and color coding indicates the source publication. The organism identity is numbered as follows: (1) *Pseudomonas* sp. 17-12; (2) *Nocardia* sp. 17-3; (3) *Escherichia coli* W3110; (4) *Escherichia coli* MG1655; (5) *Methylobacterium mesophilicum* Ch8; (6) *Methylobacterium extorquens* 19ch; (7) *Kocuria erythromyxa* G38; (8) *Methylobacterium mesophilicum* 8-18; (9) *Rhodococcus rhodochorous;* (10) *Kocuria rosea* C2; (11) *Methylobacterium extorquens* B9; (12) *Halobacterium* sp. strain NRC1; (13) *Bacillus subtilis;* (14) *Bacillus subtilis* 28-95; (15) *Deinococcus radiodurans;* (16) *Deinococcus grandis* G8; (17) *Deinococcus radiodurans* R1; (18) *Deinococcus maricopensis* LB-34; (19) *Deinococcus grandis* X2; (20) *Deinococcus radiodurans* R1; (21) *Deinococcus* PO-04-20-132T; (22) *Deinococcus* PO-04-19-125T; (23) *Deinococcus* ME-04-01-32T; (24) *Deinococcus* ME-04-04-52T; (25) *Deinococcus radiodurans* R1. (Color graphics available at www.liebertonline.com/ast)

desiccation resistance as a D_{10} value or provided a full survival plot, from which D_{10} could be extracted, as this represents a comparable metric to that most commonly used for radiation resistance. The outcome is that out of the initial 25 data sets found in the literature, only 9 are directly comparable. These are shown in Table 2, listing the desiccation D_{10} and radiation D_{10} values extracted for the named organisms. The number label relates to the identification of the organism survival responses plotted in Figs. 6 and 7.

To investigate the nature of the correlation between desiccation and radiation resistance, the D_{10} matrices for desiccation and irradiation survival are plotted against each other for the 16 microorganisms recovered by meta-analysis and from this study (shown in Fig. 8). Organisms characterized in this present study are displayed as black triangles with letter labels in the figure caption. The irradiation D_{10} value at −79°C for the radiation-resistant *D. radiodurans*, $y = 50$ kGy (Richmond *et al.,* 1999), lies so far beyond the other organisms featured that this data point was not put within the plotted range. It is, nevertheless, included in the fitted trend line. The comparable data extracted from the literature review are plotted on the same axes (using the same publication pattern/color coding and organism number labeling as Figs. 6 and 7). All the studies used a cobalt-60 gamma-ray source to expose the microbes to ionizing radiation and similar desiccation resistance tests with $CaSO_4$ (Kottemann *et al.,* 2005; Rainey *et al.,* 2005; Slade and Radman, 2011), SiO_2 (Callegan *et al.,* 2008), and $CaCl_2$ (Shukla *et al.,* 2007; this present study) as the desiccant.

4. Discussion

4.1. Radiation exposure as a preculture screening method

In this study, MDV soil samples were used in an attempt to find novel radiation-resistant strains by using irradiation preselection and MDV environmental preselection for desiccation-resistant microbes in the MDV soils. The harsh environmental conditions of this coldest hyper-arid desert on Earth (as detailed in the introduction) pose extreme stresses on the microorganisms in the soils, which probably provided a selection for desiccation resistance in MDV soil

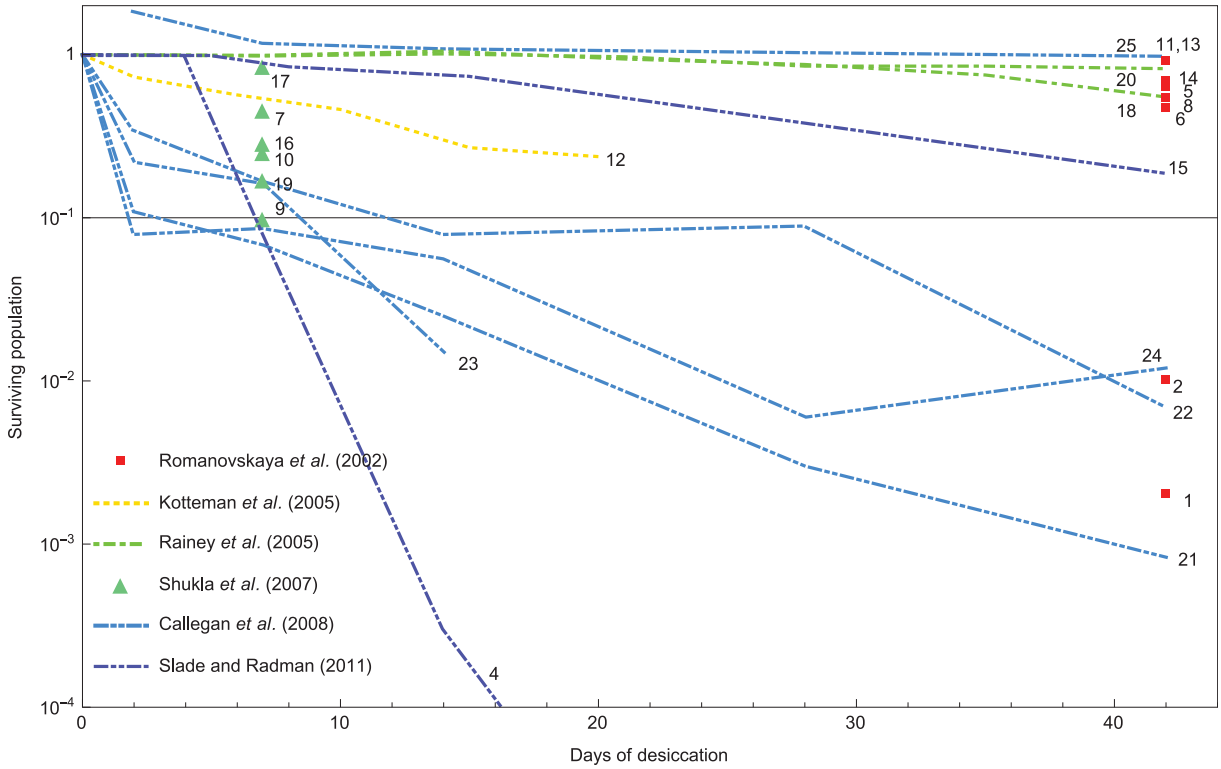

FIG. 7. Desiccation survival data for a variety of microorganisms extracted from the literature meta-analysis. The D_{10} survival threshold is indicated with a thick horizontal line at 10% remaining population. Some publications provide full response curves, but many report only the remaining viable population after 7 or 42 days (appearing as vertical arrays of data points). Pattern and color coding indicate the source publication, and organism identity is numbered following that of Fig. 6. The precise survival data for *Escherichia coli* W3110 (point 3) was not reported in Shukla *et al.* (2007) and it is therefore not shown in this figure. (Color graphics available at www.liebertonline.com/ast)

microorganisms. In turn, the cellular biology conferring desiccation resistance is thought to incidentally confer radiation resistance (as explained in the introduction) in the microbes, providing a further selective effect for radiation-resistant strains. Indeed, desiccation-resistant microbes have been previously found in the MDV (Cowan *et al.,* 2002; Pointing *et al.,* 2009; Cary *et al.,* 2010; Dartnell *et al.,* 2010a), and both desiccation and radiation-resistant microorganisms have been previously isolated from the MDV (Billi *et al.,* 2000; Hirsch *et al.,* 2004).

Furthermore, gamma radiation was used to simulate the martian ionizing radiation environment because (1) gamma rays can uniformly irradiate soil sample target volumes to a well-known total dose needed for the preselection experiments here (unlike high-energy particle accelerators whose narrow beams are not able to uniformly irradiate the bulk soil samples required in this study and only emulate a small component of the complete martian cosmic ray environment) and (2) samples could be exposed to high ionizing radiation doses of up to tens of kilograys within a few hours

Table 2. Desiccation and Radiation Survival Data Extracted from Literature Meta-Analysis

Organism	*Label*	*Desiccation D_{10} (days)*	*Radiation D_{10} (kGy)*	*Reference*
Halobacterium sp. strain NRC1	12	27*	5.0	Kottemann *et al.,* 2005
Deinococcus maricopensis LB-34	18	62*	11.5	Rainey *et al.,* 2005
Rhodococcus rhodochorous	9	7.0	1.0	Shukla *et al.,* 2007
Deinococcus PO-04-19-125T	22	11.7	3.6	Callegan *et al.,* 2008
Deinococcus PO-04-20-132T	21	8.7	2.2	
Deinococcus ME-04-01-32T	23	2.0	3.8	
Deinococcus ME-04-04-52T	24	1.8	4.0	
Deinococcus radiodurans	15	53*	11.6	Slade and Radman, 2011
Escherichia coli MG1655	4	6.6	1.04	

Only papers that reported both desiccation and radiation survival data for the same organism in a comparable form were selected. D_{10} values were either stated explicitly in the referenced paper or readily determinable from the presented survival plot. Number labels correspond to those for data points in Figs. 6 and 7.

*Value obtained by a small extrapolation from provided data.

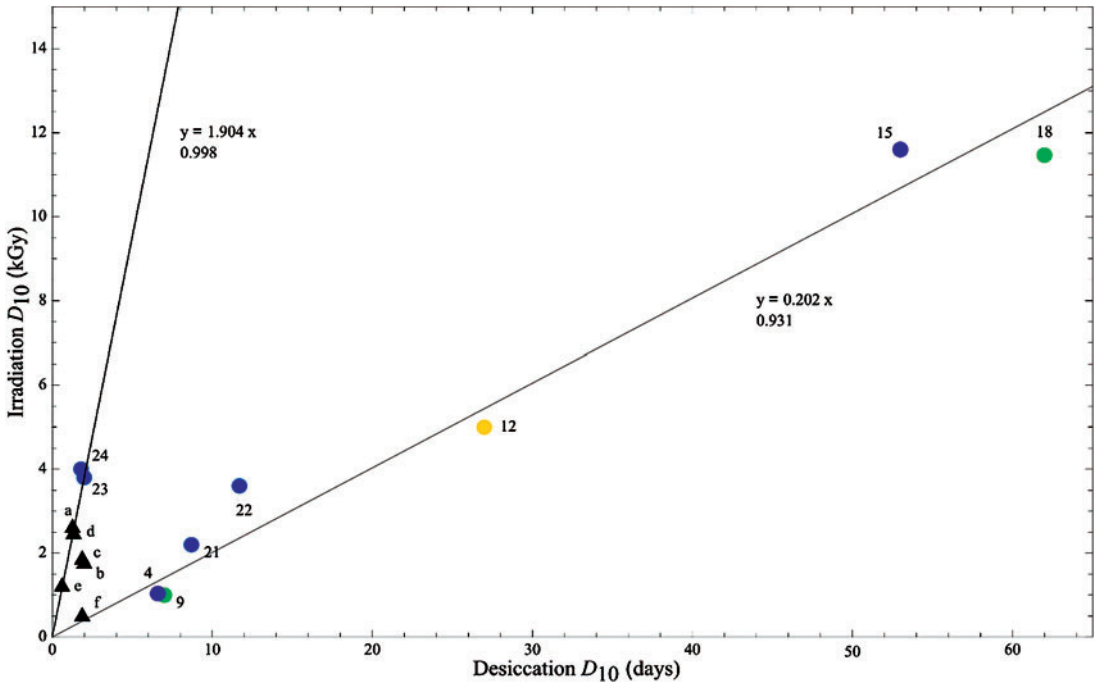

FIG. 8. Ionizing radiation survival D_{10} values plotted against the corresponding desiccation resistance D_{10} metric for 16 microorganisms (from this study and from the literature analysis). Strains characterized in this present study are shown as black triangles and identified by a letter label as listed in Table 1. The D_{10} value for *Deinococcus radiodurans* irradiated at −79°C (g) lies beyond the plotted range at (26, 50), but it is included in the fitted trend line. Desiccation and radiation survival data extracted from the literature review appear as colored dots. The organisms are identified by a number label (indicated in Table 2, Figs. 6 and 7) and by the same color coding as in Figs. 6 and 7. Radiation experiments from the literature generally irradiated bacterial samples at RT or chilled. Thus, due to the temperature dependence of irradiation survival, they lie on a different trend line to the exposures here conducted at −79°C. The equation and Pearson correlation coefficient of each trend line are labeled. (Color graphics available at www.liebertonline.com/ast)

(as opposed to thousands of years needed to accumulate such a dose during exposure in space) (Dartnell *et al.,* 2010b). Gamma rays are commonly used in space radiobiology studies, as they offer a high flux, high penetration, and practicality of experimentation, even though the ionization they cause does not fully recreate the effects of accelerated ions on the surface and in the subsurface of Mars [discussed in detail in Dartnell *et al.,* (2010b)]. Moreover, the high-charge/high-energy ions get fragmented or are absorbed by 2 m depth; therefore the radiation environment at greater depth is dominated by low linear energy transfer radiation, which can be more faithfully emulated by using gamma rays (Dartnell *et al.,* 2007a, 2010b). Other studies that have previously used gamma rays as a source of ionizing radiation to simulate the martian cosmic ray environment include Kminek *et al.* (2003), Kminek and Bada (2006), Moeller *et al.* (2010), and Quinn *et al.* (2013). Since the purpose of this simulation study was to simulate accumulating radiation damage in dormant or cryopreserved martian life for thousands to millions of years, doses up to 15 kGy of gamma radiation were used in these experiments.

A radiation-resistant fraction of the initial microbial community was selected by exposing soil samples to 4 and 6 kGy doses of ionizing radiation, before attempting to culture survivors (Fig. 2). Cells remaining viable in the sample will have endured the radiation exposure, and this community of survivors should have contained strains possessing high radiation resistance. This expectation was later checked by performing the second irradiation experiment: a full radiation survival test on the novel strains isolated from the preselection procedure. The success of the preselection for radiation-resistant microbes within the Antarctic soil community is clear from Fig. 4. The least radiation resistant of the *Halomonas* novel isolates, strain MVT 463, exhibited over 2 orders of magnitude greater survival after 10 kGy than *Brevundimonas* sp. MV7, previously isolated without preselection. For *Halomonas* sp. MVT 161 the difference is more than 4 orders of magnitude.

The radiation-surviving strains, identified by 16S rRNA gene sequencing, belong to five bacterial genera as follows: *Rhodobacter*, *Herbaspirillum*, *Hymenobacter*, *Staphylococcus*, and *Halomonas* (Fig. 3). The single *Rhodobacter* isolate is related to purple photosynthetic bacteria that are able to fix nitrogen, which would equip cells as competent primary producers in the oligotrophic MDV environment. Radiation-resistant relatives of the *Herbaspirillum* genus, from Tataouine desert sand, were also isolated by preselection for ionizing radiation resistance (Chanal *et al.,* 2006). *Hymenobacter* has already been identified as a genus containing ionizing radiation–resistant species, such as those isolated from irradiated pork (Collins *et al.,* 2000). Both Rainey *et al.* (2005) and Zhang *et al.* (2007) isolated new strains of this genus from desert soil or sand samples using an ionizing radiation exposure preculturing screening

method analogous to that employed in this study. One isolate was identified to be a species of *Staphyloccocus*. Strains of *Staphylocci*, *Herbaspirillum,* and *Hymenobacter* have been found in the extreme desiccating, oligotrophic, and artificial environments of several spacecraft assembly clean rooms (Venkateswaran *et al.,* 2003; Moissl *et al.,* 2007; Vaishampayan *et al.,* 2010). These strains are therefore of crucial importance to astrobiology, as they represent terrestrial microbes likely to contaminate Mars during robotic (or human) space exploration, if they were to survive the planetary protection guidelines currently in place (Rummel *et al.,* 2002; COSPAR, 2011) and the interplanetary transit to Mars. The resistance of terrestrial microorganisms during a hypothetical journey to Mars has been studied previously, for example during the EXPOSE-E mission (Horneck *et al.,* 2012; Moeller *et al.,* 2012; Nicholson *et al.,* 2012).

Halomonas strains, however, have not been previously investigated in terms of ionizing radiation resistance, which is why they were selected here for further study. The *Halomonas* genus has been cultured previously from very saline habitats, which may provide it with the necessary cellular repair mechanisms for desiccation tolerance and therefore radiation resistance (Kottemann *et al.,* 2005). Although all four strains showed close 16S rRNA gene similarity, *Halomonas* sp. MVT 463 and 464 were selected to compare against 468 as they all were cultured from the same sampling site (MVT4; exposed, dry, and fine gravel at the base of the valley wall) but exhibited different colony morphology (colored pink and yellow, respectively). *Halomonas* sp. MVT 161 was selected as it exhibited an indistinguishable colony morphology to 468, but it was cultured from a very different habitat (MVT1; wet ground in a floodplain). To our knowledge, only one other study has performed desiccation assays on *Halomonas* species [*Halomonas variabilis* (Burch *et al.,* 2013)] but no radiation resistance tests. These authors found that *H. variabilis* experienced a >log2 reduction in survival after 6 h and ~Log3 reduction after 1 day, compared to the isolates in this study exhibiting ~log1 reduction after 2 days. Therefore, *H. variabilis* strains studied by Burch *et al.* (2013) were significantly less resistant to desiccation than the *Halomonas* strains preselected from radiation resistance in this study. In the future, the desiccation and radiation resistance properties of the four *Halomonas* strains studied here should be compared to those of other species of halomonads, for example from a milder climate. The characteristics of the entire group of microorganisms could be tested for in this way in order to determine whether the survival in the MDV does indeed provide halomonads with greater desiccation and radiation resistance properties. The other strains isolated from the MDV should also be tested for those two properties. Furthermore, future experiments should compare the desiccation and incidental radiation resistance of these microbes pre and post preselecting them for desiccation and radiation resistance. This will allow for the determination of the influence of the MDV habitat alone on these properties of the microorganisms.

4.2. Link between desiccation and radiation resistance

The same type of repair mechanisms for recovery from desiccation DNA damage are hypothesized to be used by cells to recover from ionizing radiation–induced DNA damage (Mattimore and Battista, 1996; Billi *et al.,* 2000). Extreme radiation resistance is, therefore, not believed to be an adaptive strategy itself but a consequence of natural selection for desiccation survival (Mattimore and Battista, 1996). The nature of the proposed correlation between desiccation and radiation resistance has not previously been quantitatively assessed for a broad diversity of microorganisms. In this study, the correlation between the survival responses to desiccation and radiation exposure was studied for the same strains (Figs. 4 and 5). A meta-analysis of previously published data in the literature was also conducted to gather a greater data set. Only studies that reported both desiccation and ionizing radiation survival experiments on the same microorganism were included (Figs. 6–8).

Several observations can be made from the survival responses of different organisms represented in Fig. 8. Points 21 to 24 are all clustered relatively closely together in this desiccation/radiation resistance space. These are the psychrophilic, but radiation sensitive, new *Deinococcus* strains isolated by Callegan *et al.* (2008) from high-altitude (>3500 m) alpine environments, which were also found to be desiccation-sensitive and UV-sensitive. Their reduced resistance to both desiccation and radiation is clear in the Fig. 8 plot, with these strains segregating far away from the other *Deinococcus* species, *D. radiodurans,* point 15 (Slade and Radman, 2011), and *D. maricopensis,* point 18 (Rainey *et al.,* 2005). The very similar survival characteristics of two unrelated organisms from separate studies, *Escherichia coli* [4 (Slade and Radman, 2011)] and *Rhodococcus rhodochorous* [9 (Shukla *et al.,* 2007)], are also immediately apparent.

Separate trend lines were fitted to the two sets of results—the sensitivity experiments conducted in this present study and those collected from appropriate reports in the literature. This is due to the two different conditions used for the radiation exposures. The irradiations reported here were conducted frozen at −79°C on dry ice. This was planned for two reasons: to completely suspend cellular metabolism and damage repair during irradiation and to emulate martian surface conditions in the interests of astrobiology. Temperature during exposure is known to affect survival through the minimization of indirect radiation damage (Powers and Tallentire, 1968; Dartnell *et al.,* 2010a, 2010b). In this way, the radiation survival data collected here [as well as that reported by Richmond *et al.* (1999)] cannot be directly compared to that generated by irradiation of samples at RT or chilled on ice, as is the case for the results extracted from the other publications. This minimization of radiation damage at very low temperatures means that the gradient of the fitted trend line for the −79°C frozen irradiation exposures (this study) is steeper than that of the RT or chilled exposures (literature data).

A clear correlation between the D_{10} values of the desiccation and ionizing radiation resistance for both irradiation experimental regimes (with regard to temperature) can be seen in Fig. 8. These fitted trend lines reveal that, generally, an increase in the desiccation resistance of 5 days corresponds to an increase in RT radiation resistance of 1 kGy (literature data). An increase in desiccation resistance of just over half a day corresponds to an increase in radiation resistance of 1 kGy when irradiated frozen at −79°C. This correlation was expected from

the hypothesis that radiation resistance is a secondary consequence of adaptation to desiccation survival. Nevertheless, it had not been quantified before for a range of organisms (from this study and from the literature analysis). Freezing at −79°C, therefore, hypothetically increases the average bacterial radiation resistance by approximately 9-fold. By way of comparison, Thayer and Boyd (2001) reported an approximately 2-fold enhancement of radiation resistance of *Escherichia coli* O157:H7 and *Staphylococcus aureus* when exposed at −20°C rather than 0°C.

5. Conclusions

This is the first study to successfully isolate radiation-resistant microbes from the Mars analog MDV using gamma-ray exposure preselection. The most numerous survivors of the highest irradiation exposures were halomonads, which were studied here for the first time for both their desiccation and irradiation survival characteristics. This study also performed the first quantitative assessment of the association between desiccation and radiation resistance for a broad diversity of microorganisms (novel strains isolated in this study and from the literature analysis). A strong correlation was found between desiccation and radiation resistance, indicating that an increase in the desiccation resistance of 5 days corresponds to an increase in the RT irradiation survival of 1 kGy. Irradiation at −79°C (representative of average martian surface temperatures) increases the microbial radiation resistance 9-fold. The cold-, desiccation-, and radiation-resistant organisms isolated here therefore represent important extremophiles for understanding the potential survival of dormant or cryopreserved life in the cold desert of the martian surface.

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Author Disclosure Statement

No competing financial interests exist.

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Abbreviations Used

CFU = colony-forming unit
MDV = McMurdo Dry Valleys
MVT = Miers Valley Transect
NB = nutrient broth
PBS = phosphate buffer solution
PCR = polymerase chain reaction
RDP = Ribosomal Database Project
RT = room temperature