

ORIGINAL ARTICLE

Protein oxidation: key to bacterial desiccation resistance?

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For extremely ionizing radiation-resistant bacteria, survival has been attributed to protection of proteins from oxidative damage during irradiation, with the result that repair systems survive and function with far greater efficiency during recovery than in sensitive bacteria. Here we examined the relationship between survival of dry-climate soil bacteria and the level of cellular protein oxidation induced by desiccation. Bacteria were isolated from surface soils of the shrub-steppe of the US Department of Energy's Hanford Site in Washington State. A total of 63 isolates were used for phylogenetic analysis. The majority of isolates were closely related to members of the genus *Deinococcus*, with *Chelatococcus*, *Methylobacterium* and *Bosea* also among the genera identified. Desiccation-resistant isolates accumulated high intracellular manganese and low iron concentrations compared to sensitive bacteria. *In vivo*, proteins of desiccation-resistant bacteria were protected from oxidative modifications that introduce carbonyl groups in sensitive bacteria during drying. We present the case that survival of bacteria that inhabit dry-climate soils are highly dependent on mechanisms, which limit protein oxidation during dehydration.

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Introduction

A close relationship between bacterial desiccation tolerance and ionizing radiation (IR) resistance has been established for over three decades, but the mutual nature of the mechanisms underlying these traits remains unknown. Early on, resistance to desiccation was utilized in the selection of radioresistant bacteria from nonirradiated sources (Sanders and Maxcy, 1979). Later, radioactive environments were found to be enriched in bacterial groups known for their desiccation resistance (Fredrickson *et al.*, 2004). However, in general the vast majority of bacteria on earth are sensitive to IR, which is key to the widespread success of industrial IR-based sterilization technologies.

The molecular mechanisms underlying desiccation resistance remain poorly defined. During dehydration, bacterial cells accumulate DNA damage (Dose *et al.*, 1992). In the extremely IR- and desiccation-resistant bacterium *Deinococcus radiodurans*, for example, dehydration for 6 weeks causes approximately 60 DNA double-strand breaks (DSB) per genome, and recovery is dependent on incubation in growth medium (Mattimore and Battista, 1996). The role of DSB repair in bacterial desiccation resistance is not unlike that ascribed to DNA repair following IR, where DNA repair mutants render cells sensitive to desiccation, and survival is inextricably linked to genome reassembly (Mattimore and Battista, 1996). For any DNA-damaging condition, the functionality of DNA repair and replication systems ultimately determines whether a bacterial cell lives or dies (Minton, 1996; Makarova *et al.*, 2001).

Two independent analyses of gene induction in *D. radiodurans* that have been reported include, one recovering from desiccation (Tanaka *et al.*, 2004), the other from IR (Liu *et al.*, 2003). Both revealed

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numerous genes that were upregulated during recovery, many of which were viewed as plausible candidates for a significant role in resistance (Liu *et al.*, 2003; Tanaka *et al.*, 2004; Makarova *et al.*, 2007). The hierarchy of induced genes in both transcriptome analyses was very similar, which supported the hypothesis that the etiological radicals underlying toxicity, and cellular targets eliciting the responses in *D. radiodurans* following dehydration or irradiation were closely related. Similar to current models of IR resistance, desiccation resistance hypotheses aim to reconcile the seemingly paradoxical findings that DNA repair proteins in *D. radiodurans* function extremely efficiently, yet appear structurally unremarkable (Makarova *et al.*, 2001; Daly *et al.*, 2004; Daly *et al.*, 2007). Furthermore, most of the amino-acid residues that are distinct in *Deinococcus* and could be responsible for the structural and functional differences between the DNA repair proteins of *Deinococcus* and evolutionarily distinct bacteria are present in the DNA repair protein sequences of *Thermus* species, which are radiation sensitive but belong to the same branch of bacteria called the *Thermus-Deinococcus* group (Omelchenko *et al.*, 2005; Makarova *et al.*, 2007).

For a group of phylogenetically diverse bacteria with extreme differences in IR resistance, a correlative relationship was recently reported between intracellular manganese/iron (Mn/Fe) concentration ratios and survival, where very high and low Mn/Fe ratios corresponded to very high and low resistances, respectively. Bacteria with very different Mn/Fe ratios, however, were equally susceptible to IR-induced DNA DSB damage (Gerard *et al.*, 2001; Daly *et al.*, 2004). Instead, bacterial radioresistance and Mn/Fe ratios were quantifiably related to the level of oxidative protein damage caused during irradiation (Daly *et al.*, 2007). For Fe-rich and Mn-poor bacteria, radiosensitivity was attributed to high levels of Fe-dependent protein damage caused during irradiation (Daly *et al.*, 2007). Within this conceptual framework, we examined the levels of dehydration-induced protein oxidation in naturally desiccation-resistant bacteria isolated from shrub-steppe surface soils of south-central Washington to probe the relationship between resistance to desiccation and the extent of protein oxidation.

Materials and methods

Site description and soil sampling

Surface soil samples were collected in December 2000 along an approximately 32 km SE to NW transect between the Hanford Site 300 and 200 areas in south-central Washington. Six sites, evenly distributed along this transect, were sampled for soil from a depth of 0 to 20 cm. Vegetation was generally sparse and consisted of bunchgrass and sagebrush. Soil moisture contents at the time of

collection ranged from 1.3% to 6.3% by weight as determined by gravimetric analysis.

Isolation of bacteria from irradiated soil

Fifty grams of each of the dry soil were placed in sterile 120-ml plastic containers and subsequently received 10, 15 or 25 kGy of IR in a ^{60}Co irradiator (MDS Nordion Inc., Kanata, Ontario, Canada). Aliquots (5 g) of irradiated soil were transferred to separate containers and 45 ml of sterile sodium pyrophosphate solution (0.1%, pH 7) was added. The soil suspensions were shaken vigorously for 30 min and a series of 10-fold dilutions were prepared from the soil suspensions in pH 7 phosphate-buffered saline (PBS, Na_2HPO_4 , 0.22%; NaH_2PO_4 , 0.022% and NaCl 0.085%). Soil suspensions and subsequent dilutions were spread onto TGY agar plates (Bacto Tryptone 1%; Yeast extract 0.5%; glucose 0.1% and Bacto agar 1.5%) in triplicate and incubated in the dark at room temperature for up to 3 weeks. The plates were periodically examined for colony development, and arising pigmented colonies were streak purified on fresh TGY agar plates and examined by light microscopy to determine cell morphology. Hanford soil isolates obtained in this manner were subsequently cultured in TGY or modified PTYG broth (30 °C, 100 r.p.m.) to a mid-log phase and preserved by freezing at -80 °C in 40% glycerol. Modified PTYG (mPTYG) consists of 0.5% peptone, 0.3% yeast extract, 0.1% glucose, 0.06% MgSO_4 and 0.007% CaCl_2 (Fredrickson and Balkwill, 1998).

16S rRNA gene phylogeny

Genomic DNA was isolated from the bacterial strains with the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA). Twenty to 100 ng of genomic DNA was used as a template for PCR amplification of an approximately 1500-base segment of the 16S rRNA gene. The PCR primers were 27F (5'-AGAGTTTGATCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') (Lane, 1991), and the resulting PCR products were purified with the QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions, except that the PCR product was eluted in 30 μl instead of 50 μl . The purified PCR products were sequenced with an ABI 3130xl Genetic Analyzer with Big Dye Terminator version 3.1 sequencing method. The sequencing primers were 529F (5'-GTGCCAGCAGCCGCG-3') (Hayashi *et al.*, 2004), G (5'-CCAGGGTATCTAATCCTGTT-3') (Balkwill *et al.*, 1997), and 1392R (5'-ACGGGCGGTGTGTRC-3') (Lane, 1991). The sequences were then assembled with Sequencer version 4.5 (Gene Codes Corporation).

The 16S rRNA sequences obtained from the 16S rDNA sequences determined as described above were aligned with 16S rRNA sequences from representative species within the *Deinococcus* line

of descent and from several related genera. Sequences of the reference strains were obtained from the Ribosomal Database Project-II (Cole *et al.*, 2007). Approximately 1400 bases were included in the phylogenetic analysis, which was performed with the PAUP computer program (Swofford, 2003), using the distance matrix option and the Jukes and Cantor (1969) distance correction method. GenBank accession numbers for the strains isolated as part of this study are: 6A2, EU029121; 1A2, EU029122; 1A3, EU029123; 1A7, EU029124; 1B1, EU029125; 1B3, EU029126; 2A2, EU029127; 2A5, EU029128; 3A1, EU029129; 3A5, EU029130; 4B4, EU029131; 4B6, EU029132; 5A4, EU029133; 5A5, EU029134; 6A4-1, EU029135; 6A4-2, EU029136; 4A4, EU308573; 4A6, EU308574; 1A1, EU308575; 1A6, EU308576 and 3B1, EU308577.

IR and desiccation resistance

Isolates analyzed for resistance to IR were grouped on the basis of phylogenetic affiliation, emphasizing strains related to *Deinococcus* but including a few other phylogenetic affiliations for comparative purposes. Cultures were grown in TGY broth (50 ml, 32 °C, 100 r.p.m.) to an OD₆₀₀ of approximately 0.5 (mid-logarithmic phase), and 10 ml of each culture was dispensed into 15 ml sterile conical polypropylene tubes. Duplicate culture tubes were irradiated to the indicated doses in the ⁶⁰Co irradiator. A single unexposed tube served as a non-irradiated control. Following irradiation, the population of surviving cells was enumerated by dilution plate counting on TGY agar as described previously (Daly *et al.*, 2004). For full IR survival profiles, the indicated strains were inoculated in liquid TGY at ~1 × 10⁶ colony-forming units per ml and grown to an OD₆₀₀ of ~0.9. Cells were then irradiated without change of broth on ice with ⁶⁰Co at 8 kGy h⁻¹ (⁶⁰Co Gammacell irradiation unit, JL Shepard and Associates, Model 109). At the indicated doses, cultures were appropriately diluted and plated on TGY agar medium, and colony-forming unit counts were determined after 5–7 days' incubation at 32 °C. The surviving population is expressed as the log of the ratio of cells surviving a given acute dose relative to the unexposed control.

For desiccation resistance experiments, cultures representing a range of IR resistance were grown in 5 ml TGY broth at 32 °C until the late-stationary phase. One milliliter of each culture (~5 × 10⁹ cells) was harvested by centrifugation, the supernatant discarded, and the cell pellet resuspended in 1 ml of sterile water which had been pre-equilibrated with Hanford soils as follows: 4 g of Hanford soil, previously sterilized by autoclaving, was mixed with 40 ml of sterile water. Aliquots (25 µl) (~1 × 10⁸ cells) of the final cell suspensions were added to individual wells of standard 96-well plates. The microtiter plates were placed in a desiccator containing anhydrous CaSO₄ (WA

Hammond Drierite Co., Xenia, OH, USA), which maintained the relative humidity at approximately 17%. The desiccator was maintained at room temperature. After 1, 2, 3 and 4 weeks, a microtiter plate was removed and 250 µl of sterile water were added to individual wells to resuspend the desiccated cells. For each desiccation dose, three independent wells for a given strain were enumerated. Appropriate dilutions were made in sterile water and subsequently plated on TGY agar. The surviving population is expressed as the log of the ratio of cells surviving a given period of desiccation relative to viable cell count of the original inocula.

Oxidative protein damage following desiccation

For desiccation, the indicated strains were cultured aerobically in TGY at 32 °C to the late-logarithmic stage. For each culture, 150 ml were harvested by centrifugation, resuspended in 30 ml 50 mM, pH 7.0 potassium phosphate buffer (PPB), washed in PPB twice and finally resuspended in 3 ml PPB. These cell suspensions, containing between 10¹⁰ and 10¹¹ cells, were transferred to individual wells of microtiter plates (Tissue Culture Plate, six-well, Becton Dickinson Labware, Bedford, MA, USA). The microtiter plates were centrifuged at 2000 g at 4 °C for 20 min and the supernatants removed. Unsealed microtiter plates were placed in desiccators with Drierite (~17% relative humidity) and stored at room temperature for 6 days. After 6 days of desiccation, cells were resuspended in 2.5 ml of 50 mM PPB at room temperature for 30 min and then placed on ice. Cell suspensions were adjusted to 1% (v/v) β-mercaptoethanol and passed through a French pressure cell (0 °C) at 20 000 lb/in², and the lysate was centrifuged twice at 12 000 g at 4 °C for 30 min. The protein concentration of a supernatant was determined by the Coomassie assay (BioRad, Hercules, CA, USA).

Protein oxidation was measured using OxyBlot Oxidation Detection Kit (catalog no. S7150, Chemicon International, Temecula, CA, USA), including the indicated molecular weight markers (O of Figure 4). The carbonyl groups in oxidized protein side chains were derivatized to 2,4-dinitrophenylhydrazones by reaction with 2,4-dinitrophenylhydrazine (DNPH) for 15 min in 3% (w/v) SDS. Western blotting: the DNP-derivatized protein samples were separated by polyacrylamide denaturing gel electrophoresis (4%–20% gradient gels; BioRad) at 200 V for 50 min followed by transferring proteins to a nitrocellulose membrane (BioRad) for 40 min. The membranes were incubated with primary antibody, specific to the DNP moiety of the proteins. This step was followed by incubation with a horse-radish peroxidase antibody conjugate directed against the primary antibody (secondary antibody: goat anti-rabbit IgG). The membranes were then treated with chemiluminescent SuperSignal

substrate (Pierce, Rockford, IL, USA) and imaged by exposure to light sensitive films (BIOMAX Light Film, Kodak, Rochester, NY, USA). Carbonylation in relative units was quantified using a FUJIFILM Luminescent Image Analyzer LAS-1000plus and Image Reader LAS-1000 Life (FUJI Medical Systems USA, Stamford, CT, USA). To measure the intensity of the individual lanes, the intensity profile of each lane was generated from the digitized gel image using Image Gauge software (FUJI Medical Systems USA).

Intracellular Mn and Fe

The protocol for determining intracellular Mn and Fe concentrations was identical to that previously reported (Daly *et al.*, 2004). For these experiments, all Hanford soil isolates were grown in mPTYG. Each culture, in triplicate, was grown in 100 ml medium to the late logarithmic phase (~ 1.0 OD₆₀₀). Cells were harvested by centrifugation (10 000 g, 10 min) and were washed twice with an equal volume of PBS, followed by an additional wash with PBS containing 1 mM ethylenediaminetetraacetic acid to remove metals loosely bound to the cell envelope, and then resuspended in 11 ml PBS in Teflon centrifuge tubes. One milliliter of this cell suspension was removed and used to determine protein concentration. The remaining cells were once again centrifuged and used for the analysis of Mn and Fe. The cell pellets were digested in 2 ml of concentrated HNO₃ (Fisher Optima with certificate of analysis, catalog no. A467, Fe < 10 and Mn < 2 parts per trillion) for 1 h in an 80 °C water bath. The sample digest was then diluted with ultrapure water to a final HNO₃ concentration of 2%. These samples were analyzed for Mn and Fe by inductively coupled plasma-mass spectrometry (ICP-MS) (model 4500, Agilent Technologies, Palo Alto, CA, USA). To avoid metal contamination, all lab wares used for these analyses were composed of plastic or Teflon and acid rinsed before use. Ultrapure water with a resistivity of up to 18.2 Megohms-cm with less than 2 p.p.b. (parts per billion) total organic carbon (NANOpure Diamond ultraviolet TOC, Barnstead International, Dubuque, IA, USA) was used throughout this experiment. A blank, prepared in the same manner but without cells, had < 20 p.p.b. Fe and < 0.1 p.p.b. Mn.

Results

Phylogeny of Hanford soil isolates

A total of 63 bacterial cultures were obtained from irradiated Hanford soils by plating soil dilutions on TGY agar and isolating pigmented colonies. For 47 of these isolates, DNA was successfully extracted, PCR amplified and sequenced for 16S rRNA gene phylogenetic analysis (Table 1; Figure 1). More than one-half of the 47 isolates had nearest GenBank

relatives who were most closely affiliated with members of the genus *Deinococcus* (Table 1). Isolates related to members of the α -proteobacteria were the next most abundant, including *Chelatococcus* (1B4, 1B7, 4A1, 4B3, 4B4 and 5A4), *Methylobacterium* (2A4 and 4A4) and *Bosea* (1B5 and 1B6). A more detailed phylogenetic analysis of strains related to *Deinococcus* revealed considerable phylogenetic diversity (Figure 1) including 19 out of 47 isolates whose nearest relative was a cloned sequence from an uncultured organism. Among these were clones (Table 1, nearest GenBank relative accession numbers beginning with DQ532) associated with samples collected from spacecraft assembly clean rooms described as highly desiccated and nutrient-poor environments (Moissl *et al.*, 2007).

Radiation and desiccation resistance

Strains representing a range of phylogenetic affiliations were characterized for their resistance to IR. Among the Hanford soil isolates, those strains whose closest identified relatives were members of the genus *Deinococcus* exhibited the greatest degree of resistance, yielding survivors following 10 or 25 kGy. Four shrub-steppe Hanford soil isolates related to *Deinococcus* (1A1, 1A6, 3B1 and 5A5) were subjected to detailed IR-resistant profiling (Figure 2). These four isolates exhibited extreme IR-resistant profiles that were similar to other *Deinococcus* species characterized previously, which typically have 10% colony forming unit survival values (D_{10}) above 10 kGy (Daly *et al.*, 2004). For example, the D_{10} values for irradiated *D. radiodurans* type strain R1 and strain 7b-1 are between 12 and 20 kGy, depending on growth stage. The type strain was isolated in 1956 (Anderson *et al.*, 1956), and strain 7b-1 was isolated in 2000 from Hanford vadose sediments contaminated with high-level nuclear waste (Fredrickson *et al.*, 2004). These levels of IR resistance are more than 200 times greater than the D_{10} value reported for the dissimilatory metal-reducing bacterium *Shewanella oneidensis* MR-1 (D_{10} , 0.07 kGy) (Daly *et al.*, 2004), whose DNA repair systems paradoxically appear quantitatively and qualitatively more complex and diverse than those reported for *D. radiodurans* (Daly *et al.*, 2004; Qiu *et al.*, 2006). Additionally, we built full IR-survival curves for α -proteobacterial isolates 4A4 and 4A6, which showed intermediate IR-resistant values (D_{10} , ~ 2 kGy) (Figure 2).

Desiccation resistance assays (Figure 3) were carried out for the same shrub-steppe strains investigated for IR resistance (Figure 2), as well as *S. oneidensis* and *D. radiodurans* strains R1 and 7b-1 (Fredrickson *et al.*, 2004) for comparison. In these experiments, cells were added to sterile Hanford soil and placed in a desiccator with anhydrous CaSO₄ for the indicated times. All

Table 1 Phylogenetic and γ -radiation resistance characteristics of isolates from Hanford soil

Soil ID	Isolate identity	Nearest GenBank relative	SimRank	Accession no. of relative	Dose ^a (kGy)	%Irradiation survival ^b	
1A	1A1	Uncultured bacterium clone JPL2-78	0.99	DQ532164	25	53.7	
	1A2	<i>Staphylococcus pasteurii</i> CV5	1.00	AJ717376	10	0.0	
	1A3	Uncultured bacterium JPL_94	0.98	DQ532147	— ^c	—	
	1A4	Uncultured bacterium clone JPL2-78	0.99	DQ532164	25	51.3	
	1A5	Uncultured bacterium clone JPL2-78	1.00	DQ532164	25	52.7	
	1A6	Uncultured bacterium clone JPL2-78	0.97	DQ532164	25	44.6	
	1A7	Uncultured bacterium JPL_93	0.98	DQ532146	25	41.0	
1B	1B1	Uncultured bacterium JPL_94	0.98	DQ532147	25	47.9	
	1B2	Uncultured bacterium clone JPL2-78	0.99	DQ532164	25	66.9	
	1B3	Uncultured bacterium JPL_94	0.99	DQ532147	25	48.0	
	1B4	<i>Chelatococcus</i> sp. 91214	0.98	DQ020478	—	—	
	1B5	<i>Bosea thiooxidans</i> isolate TJ1	0.98	AF508112	—	—	
	1B6	<i>Bosea thiooxidans</i> isolate TJ1	0.97	AF508112	—	—	
	1B7	<i>Chelatococcus</i> sp. A	0.99	DQ497243	—	—	
2A	2A2	Uncultured <i>Deinococcus</i> sp. clone K9-35	0.99	AY905383	—	—	
	2A4	<i>Methylobacterium</i> sp. AC72a	0.99	AY776209	—	—	
3A	2A5	Uncultured <i>Deinococcus</i> sp. clone K9-35	0.89	AY905383	—	—	
	3A1	Uncultured bacterium JPL_94	0.98	DQ532147	25	38.1	
	3A3	<i>Deinococcus maricopensis</i> strain KR-23	0.97	AY743276	25	32.6	
3A	3A5	<i>Deinococcus maricopensis</i> strain LB-34	0.88	AY743274	—	—	
	3A6	Uncultured soil bacterium clone TD3	0.99	DQ248308	—	—	
	3B	3B1	Uncultured bacterium clone JSC9-F	0.94	DQ532262	15	53.5
		3B3	<i>Deinococcus maricopensis</i> strain KR-23	0.97	AY743276	—	—
4A	3B4	Uncultured bacterium clone JPL2-78	0.99	DQ532164	25	46.0	
	4A1	<i>Chelatococcus</i> sp. 91214	0.98	DQ497243	—	—	
	4A2	Uncultured soil bacterium clone TD3	0.99	DQ248308	10	0.0	
	4A4	<i>Methylobacterium</i> sp. AC72a	0.98	AY776209	10	0.0	
	4A5	Uncultured soil bacterium clone TD3	0.99	DQ248308	10	27.6	
	4A6	Uncultured soil bacterium clone TD3	0.99	DQ248308	10	0.0	
4B	4B1	<i>Deinococcus maricopensis</i> strain KR-23	0.97	AY743276	20	55.5	
	4B3	<i>Chelatococcus</i> sp. 91214	0.98	DQ497243	10	33.6	
	4B4	<i>Chelatococcus</i> sp. 91214	0.93	DQ020478	10	0.0	
	4B5	Uncultured soil bacterium clone TD3	0.99	DQ248308	—	—	
	4B6	Uncultured bacterium	0.93	DQ125646	10	0.0	
	5A	5A3	Uncultured <i>Deinococcus</i> sp. clone K9-35	0.95	AY905383	15	60.7
5A4		<i>Chelatococcus asaccharovorans</i>	0.91	AY167839	—	—	
5A5		Uncultured <i>Deinococcus</i> sp. clone K9-35	0.81	AY905383	25	56.0	
6A	6A1	<i>Deinococcus sonorensis</i> strain KR-136	0.99	AY743285	25	57.4	
	6A2	<i>Hymenobacter gelurpurpurascens</i> Txg1	0.95	Y18836	—	—	
	6A3	Bacterium Ellin512	0.99	AY960775	—	—	
	6A4-1	<i>Deinococcus sonorensis</i> strain KR-89	0.97	AY743283	25	43.4	
	6A4-2	<i>Deinococcus sonorensis</i> strain KR-90	0.97	AY743284	25	47.9	
	6A6	<i>Deinococcus sonorensis</i> strain KR-136	0.99	AY743285	25	66.1	
	6A8-1	Uncultured bacterium clone JPL2-78	1.00	DQ532164	—	—	
	6A8-2	<i>Deinococcaceae</i> bacterium TDMA-uv53	0.90	AB265180	—	—	
6B	6B1	<i>Deinococcus sonorensis</i> strain KR-136	0.99	AY743285	25	58.2	
	6B3	<i>Deinococcus sonorensis</i> strain KR-136	0.99	AY743285	25	47.9	

^aDose to cultures provided via ⁶⁰Co irradiator.^bIrradiation survival % is calculated as the log ratio of cell numbers after and before irradiation.^cNot done.

Strains in bold type were included in more detailed analyses of radiation- and desiccation-resistance.

Hanford soil *Deinococcus* isolates exhibited levels of desiccation resistance as high or higher than the type-strain R1 (Figure 3). Strains 4A4 and 4A6, both α -proteobacteria, were significantly less tolerant to desiccation than any of the *Deinococcus* strains, exhibiting significant loss of viability after 1 week and decreasing further to below detection by 3 weeks, but still substantially more desiccation resistant than *S. oneidensis* and other γ -proteobacteria (Daly et al., 2004).

Protein oxidation during desiccation

Although genome damage and recovery following exposure to IR or desiccation has been examined extensively in *Deinococcus* and other bacteria over the last three decades, to our knowledge there had been no investigations in bacteria that have examined the relationship between desiccation and protein damage. In the context of recent studies, which implicated protein damage as the primary determinant of bacterial radioresistance

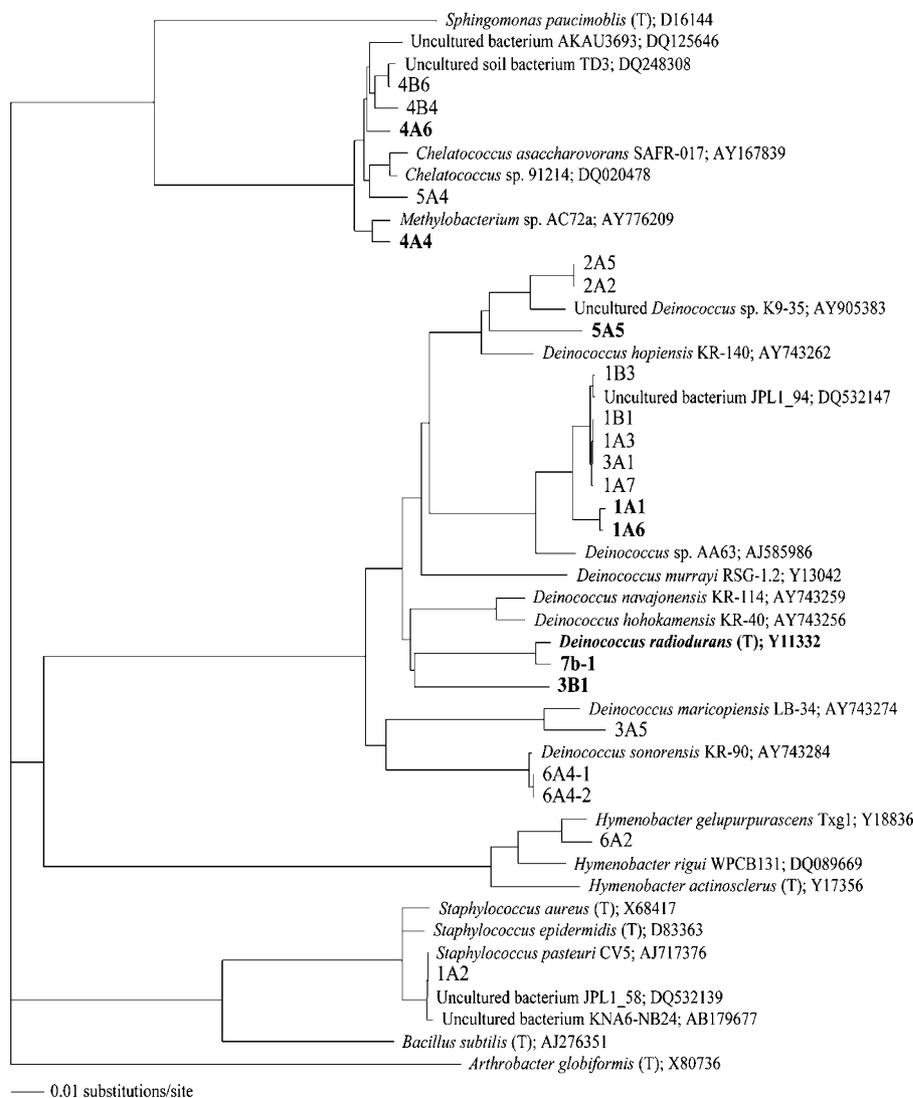


Figure 1 16S rDNA-based molecular phylogeny (distance matrix method) of bacterial isolates cultivated from irradiated Hanford soil. The scale bar shows the expected number of substitutions per sequence position. Strains in bold type were included in more detailed analyses of radiation- and desiccation-resistance.

(Daly *et al.*, 2007), we examined the level of oxidative protein damage using an assay for carbonyl group (aldehydes and ketones) generation into proteins at Lys, Arg, Pro and Thr residues (Stadtman, 1993) (Figure 4). The level of carbonyl groups in proteins is widely used as a marker of oxidative protein damage and has attracted a great deal of attention due to its irreversible and unrepairable nature (Nystrom, 2005). In phylogenetically diverse bacteria cultured in rich medium, protein carbonyl levels are typically very low or undetectable (Daly *et al.*, 2007). It is important to note that since not all oxidative modifications lead to carbonyl derivatives, the levels of carbonylation detected after desiccation using the carbonyl assay represent minimal values (Nystrom, 2005).

To evaluate the extent of protein oxidation during desiccation, cells were desiccated for 6 days. After 6 days, cell viability was determined (Supplementary Figure S1), proteins were extracted and the extent of

protein oxidation was determined by western blot immunoassay for protein-bound carbonyl groups (Figure 4). The results of the protein oxidation assay correlated with the organisms' ability to resist desiccation (Figure 3), with the *Deinococcus* species (1A1, 1A6, 3B1, 5A5, 7b-1 and R1) exhibiting the least amount of protein oxidation as determined by the relative number and intensity of bands in the carbonyl assay and subsequently quantified by densitometry (Figure 4). In contrast, α -proteobacterial isolates 4A4 and 4A6 sustained higher levels of protein oxidation during desiccation than the *Deinococcus* isolates, but substantially less than in *S. oneidensis* (Figure 4).

Intracellular Mn and Fe concentrations

Previous research has implicated intracellular accumulation of Mn(II) in facilitating the survival of *D. radiodurans* and other phylogenetically

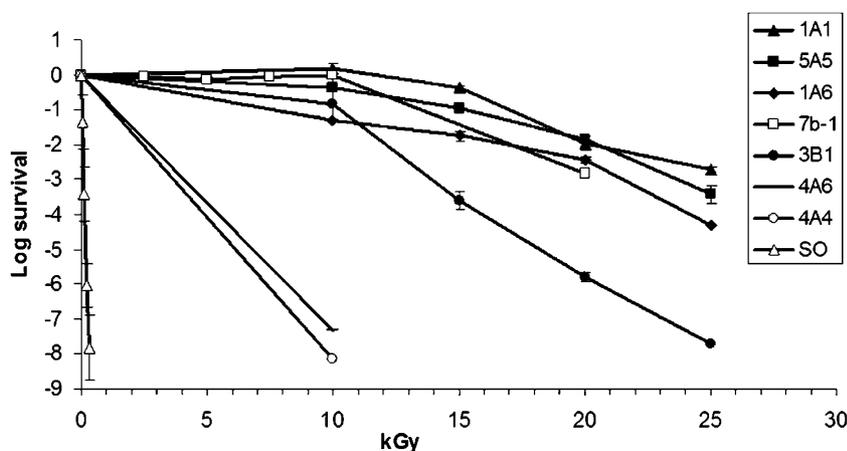


Figure 2 Survival of representative Hanford soil isolates exposed to acute IR. *D. radiodurans* strain 7b-1 was obtained from a previous investigation (Fredrickson *et al.*, 2004) and *S. oneidensis* MR-1 (SO) was included for comparative purposes.

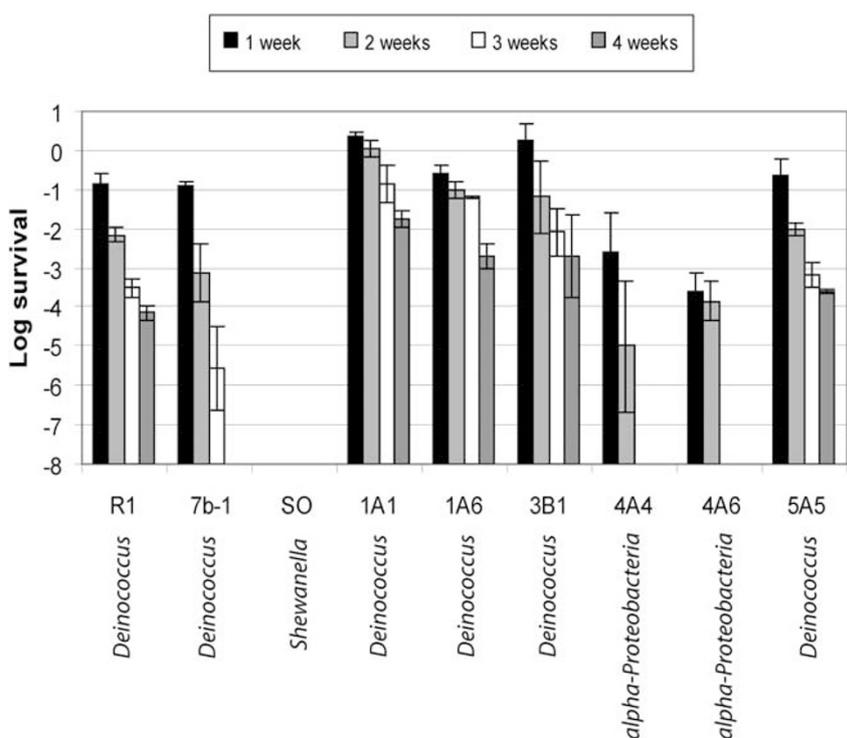


Figure 3 Survival of representative strains as determined by viable plate count after 1, 2, 3 and 4 weeks desiccation in sterile Hanford soil. The surviving population is expressed as the log of the ratio of cells surviving desiccation relative to cell population before desiccation.

distinct bacteria following exposure to high doses of acutely or chronically delivered IR (Daly *et al.*, 2004). In exploring the relationship between IR resistance and the intracellular metal contents of bacteria, a strong correlation was observed between a cell's intracellular Mn/Fe concentration ratio and its viability following irradiation (Daly *et al.*, 2004). We reasoned that the Mn/Fe ratio might be a common feature among environmentally robust bacteria, and we measured the intracellular

concentrations of these metals in the representative Hanford soil isolates (Supplementary Table S1). The results of these analyses revealed that all of the IR- and desiccation-resistant Hanford soil isolates had intracellular Mn/Fe concentration ratios that ranged between 0.12 and 0.37, values characteristic of bacteria displaying from high levels of IR resistance (for example, *Enterococcus faecium*: D_{10} , 2 kGy; Mn/Fe ratio, 0.17) (Daly *et al.*, 2004) to extremely high levels (for example, *D. radiodurans*: D_{10} ,

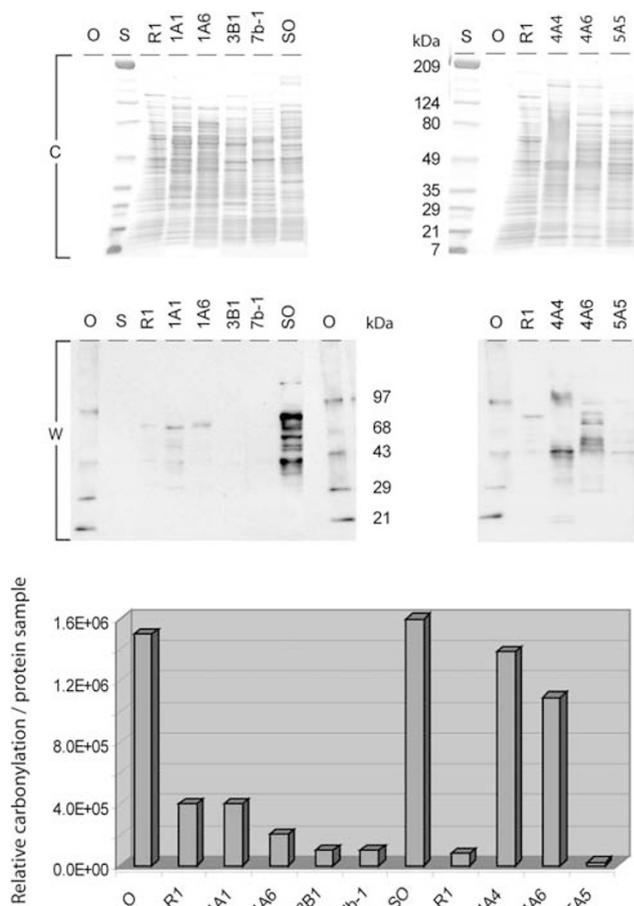


Figure 4 *In vivo* desiccation-induced oxidative protein damage. C, coomassie-stained polyacrylamide denaturing gel of 5 µg total soluble protein per lane for the indicated strains. W, western blot immunoassay of protein-bound carbonyl groups introduced into the proteins (5 µg per lane) by oxidative reactions following desiccation of the bacteria for 6 days. O, oxidized protein standards. S, wide-range protein size standards. R1, *Deinococcus radiodurans* R1 and SO, *Shewanella oneidensis* MR-1. Bottom, densitometric quantification of total protein carbonyl levels per lane (W).

16 kGy; Mn/Fe ratio, 0.24). These survival values are very high compared to IR-sensitive environmental bacteria such as *S. oneidensis* (D_{10} , 0.07 kGy; Mn/Fe ratio, 0.0005) and *Pseudomonas putida* (D_{10} , 0.25 kGy; Mn/Fe ratio, 0.0001) (Daly *et al.*, 2004).

Discussion

In this study, surface soils from the region of south-central Washington that encompasses DOE's Hanford Site served as a source of desiccation-resistant bacteria. The region is categorized as shrub-steppe and receives an average of 17.2 cm of precipitation annually. Since most of the precipitation falls between the months of October and March, the soils typically become very dry during late spring and remain so during the summer and into the fall. In the present study, soil samples were

collected in December 2000, and *Deinococcus* species were readily cultivated suggesting that they are ubiquitous microbial inhabitants (Table 1). Furthermore, numerous *Deinococcus* strains cultured from the Hanford Site were phylogenetically related to strains isolated by others from the Sonoran desert, which straddles part of the United States–Mexico border and covers large regions of Arizona and California (Rainey *et al.*, 2005). Interestingly, the same study also reported the isolation of *Methylobacterium* and *Chelatococcus* from Sonoran desert soils. Thus, the soils of the Hanford Site are representative of other North American dry-soil environments and appear well suited to isolating bacteria that are adapted to survival under conditions of severe and prolonged desiccation. Seven representative bacterial isolates from the Hanford Site were the subject of detailed desiccation and IR-resistant studies reported here.

Cellular damage during dehydration has been attributed to the formation of reactive oxygen species (ROS), particularly hydroxyl and peroxy radicals, as well as more complex and longer-lived forms derived from protein hydroperoxides (Nausier *et al.*, 2005). Morphological and physiological changes that contribute to the enhanced ROS formation and damage during dehydration include loss of control of respiratory electron transport chains; elevated exposure to atmospheric oxygen as aqueous barriers to gaseous diffusion dissipate; the inability to induce antioxidant systems as the cell goes dormant; and physical distortions of DNA within dehydrating cells as their volume shrinks (Kranmer, 2002; Kranmer and Birtic, 2005). In ROS-based toxicity models, it is often tacitly assumed that bacteria that are susceptible to DNA damage must be similarly susceptible to protein damage. That is not the case, however, as demonstrated recently for irradiated bacteria. Although the number of DNA DSBs inflicted by a given dose of IR in extremely sensitive and resistant bacteria is about the same, the levels of protein oxidation induced in sensitive cells by a given dose of IR far exceed those in resistant cells (Daly *et al.*, 2007). In this context, protein protection during irradiation in extremely resistant bacteria is positively correlated with the accumulation of intracellular Mn together with relatively low Fe levels (Daly *et al.*, 2004). *In vitro* Mn(II) ions have been shown to inhibit Fe-dependent formation of protein carbonyl groups during irradiation (Daly *et al.*, 2007); and *in vivo* protein carbonylation is widely used as a marker of irreversible and unrepairable oxidative protein damage (Nystrom, 2005).

Our findings that desiccation-induced cellular protein damage is quantifiably related to desiccation resistance and intracellular Mn/Fe concentration ratios (Figures 3 and 4, Supplementary Table S1) could help explain why bacteria that encode a similar repertoire of DNA repair functions display such large differences in desiccation resistance

(Daly *et al.*, 2004). The *Deinococcus* isolates were highly resistant to desiccation and were the least susceptible to desiccation-induced protein carbonylation; α -proteobacterial isolates 4A4 and 4A6 showed intermediate levels of desiccation resistance and desiccation-induced carbonylation; and *S. oneidensis* was the most sensitive to desiccation and highly susceptible to desiccation-induced carbonylation (Figure 4). For these bacteria, the trend in their desiccation resistance profiles mirrored their resistance to IR, as observed previously for other bacteria (Daly *et al.*, 2004). Thus, for the group of phylogenetically diverse dry-climate soil bacteria reported here, our findings support the idea that the degree of resistance is determined by the level of oxidative protein damage caused during desiccation.

The role of Mn accumulation in microbial IR resistance has been examined recently (Daly *et al.*, 2004). For a group of phylogenetically distinct model wild-type bacteria grown under standardized conditions, intracellular Mn/Fe concentration ratios were correlated with their IR resistance (Daly *et al.*, 2004). For bacteria with Mn/Fe concentration ratios equal to or below 0.0005 (for example, *S. oneidensis*), cells were extremely sensitive to IR. In contrast, bacteria with Mn/Fe concentration ratios between 0.17 and 0.46 (for example, *Enterococcus faecium* and *Deinococcus geothermalis*) were IR resistant, on the basis of their ability to survive high doses of acute IR and grow under chronic IR (Daly *et al.*, 2004). Since then, we have examined other bacteria including the IR-sensitive *Thermus thermophilus* (D_{10} , 0.8 kGy; Mn/Fe, 0.047) (Omelchenko *et al.*, 2005) and the IR-resistant archaeobacterium *Halobacterium* sp. strain NRC1 (D_{10} , 5 kGy; Mn/Fe, 0.27, unpublished). Thus, the dry-climate soil isolates reported here are well aligned with this paradigm, exhibiting high Mn/Fe concentration ratios, high levels of IR resistance, and a low susceptibility to desiccation-induced protein oxidation. *In vitro* in the presence of organic acids, orthophosphate or pyrophosphate, Mn(II) ions have been shown to be potent scavengers of superoxide (Archibald and Fridovich, 1982). On the basis of those findings, we previously proposed that Mn(II) accumulated in desiccation- and IR-resistant bacteria is complexed with organic and/or inorganic ligands (Daly *et al.*, 2007), and that the protective complexes are non-proteinaceous.

Recent whole genome comparisons between *D. radiodurans* and *D. geothermalis* have strengthened the view that deinococci rely more heavily on the high efficiency of their detoxifying systems than on the number and specificity of their DNA repair systems (Makarova *et al.*, 2007). In considering the contribution of cell cleaning to resistance, we previously showed that the IR dose dependence of bacterial cell-killing parallels protein oxidation, where sensitive bacteria were highly susceptible to IR-induced protein carbonylation, but resistant bacteria were not (Daly *et al.*, 2007). Those analyses

supported that intracellular Mn complexes in resistant bacteria scavenge a subset of IR-induced ROS, which damage proteins, but not DNA, keeping the concentration of Fe-dependent superoxide and related peroxy radicals generated during irradiation low. Results presented herein support that the mutual nature of bacterial desiccation resistance and radiation resistance resides in cytosolic Mn-dependent mechanisms that protect proteins. In our model, only when the ROS-scavenging ability of Mn-dependent systems is exhausted do the toxic byproducts of desiccation or irradiation become pervasive and inactivate DNA repair and related recovery systems (Daly *et al.*, 2007). This has the attraction of explaining the following: (i) the ability of orthologs from radiosensitive bacteria to complement *D. radiodurans* DNA repair mutants; (ii) the disparity observed between survival and DNA DSB levels in different bacteria; and (iii) the very long shoulders of desiccation-, IR- and ultraviolet-response curves for extremely resistant bacteria (Daly *et al.*, 2004; Makarova *et al.*, 2007). In comparison, models which attribute the evolution of extreme resistance in *Deinococcus* and other organisms to incremental modifications of a set of universal DNA repair genes are not easily reconciled with these characteristics (Cox and Battista, 2005; Holloman and Schirawski, 2007; Makarova *et al.*, 2007).

In conclusion, since life on earth most likely did not commonly encounter extremes of IR over geologic times (Mattimore and Battista, 1996), the extreme resistance phenotypes frequently observed in desert soil-inhabiting bacteria likely evolved in response to other forms of oxidative stress, including ultraviolet light and desiccation, as proposed previously (Mattimore and Battista, 1996). Since the bulk of soil bacteria are shielded from ultraviolet, we extend this hypothesis and propose that IR resistance of dry-climate soil bacteria is an incidental mechanism evolved to resist oxidative protein damage induced during cycles of drying and rehydration. We contend that protein oxidation is a key determinant of bacterial desiccation tolerance. Hence, these findings may come to affect models for the evolution of this complex trait with important implications for the ecology of microorganisms in natural and engineered environments where desiccation is a major stressor. We also argue that protection of proteins against ROS damage is equally or more critical for maintaining ancient bacteria in a viable state than DNA repair (Johnson *et al.*, 2007). If true, microorganisms that have developed robust systems for protecting proteins against ROS-induced damage would be expected to exhibit superior long-term cell survival.

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