Responses of Mn$^{2+}$ speciation in Deinococcus radiodurans and Escherichia coli to γ-radiation by advanced paramagnetic resonance methods

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The remarkable ability of bacterium Deinococcus radiodurans to survive extreme doses of γ-rays (12,000 Gy), 20 times greater than Escherichia coli, is undiminished by loss of Mn-dependent superoxide dismutase (SodA). D. radiodurans radiation resistance is attributed to the accumulation of low-molecular-weight (LMW) “antioxidant” Mn$^{2+}$-metabolite complexes that protect essential enzymes from oxidative damage. However, in vivo information about such complexes within D. radiodurans cells is lacking, and the idea that they can supplant reactive-oxygen-species (ROS)–scavenging enzymes remains controversial. In this report, measurements by advanced paramagnetic resonance techniques [electron-spin-echo (ESE)-EPR/electron nuclear double resonance/ESE envelope modulation (ESEEM)] reveal differential details of the in vivo Mn$^{2+}$ speciation in D. radiodurans and E. coli cells and their responses to 10 kGy γ-irradiation. The Mn$^{2+}$ of D. radiodurans exists predominantly as LMW complexes with nitrogenous metabolites and orthophosphate, with negligible EPR signal from Mn$^{3+}$ or Mn$^{4+}$. Thus, the extreme radiation resistance of D. radiodurans cells cannot be attributed to SodA. Correspondingly, 10 kGy irradiation causes no change in D. radiodurans Mn$^{2+}$ speciation, despite the paucity of holo-SodA. In contrast, the EPR signal of E. coli is dominated by signals from low-symmetry enzyme sites such as that of SodA, with a minority pool of LMW Mn$^{2+}$ complexes that show negligible coordination by nitrogenous metabolites. Nonetheless, irradiation of E. coli majorly changes LMW Mn$^{2+}$ speciation, with extensive binding of nitrogenous ligands created by irradiation. We infer that E. coli is highly susceptible to radiation-induced ROS because it lacks an adequate supply of LMW Mn antioxidants.

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ver the past 30 y, evidence has mounted for the widespread use of small manganous (Mn$^{2+}$) metabolite complexes in cellular defense against reactive oxygen species (ROS) such as superoxide (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), in addition to enzymatic mechanisms for antioxidant defense (1–14). The role of nonenzymatic defenses based on Mn is established by such findings as (i) ROS-scavenging enzymes are completely dispensable for extreme radiation resistance in Mn-accumulating prokaryotes (1, 2, 6–8, 11) and (ii) limiting Mn$^{2+}$ accumulation renders Deinococcus radiodurans cells radiation-sensitive and highly susceptible to protein oxidation (1, 9). This nonenzymatic, Mn-dependent artilllery for combating oxidative stress is manifested in cells that accumulate Mn$^{2+}$ together with various inorganic and organic ligands (2, 11, 13, 14), and has been best studied in the extremely radiation-resistant bacterium D. radiodurans (8). D. radiodurans is capable of surviving huge doses of γ-rays (12,000 Gy) 20 times greater than the bacterium Escherichia coli, and 3,000 times greater than most human cells in liquid culture (2). D. radiodurans can also endure high exposures to UV C radiation and decade-long periods of drying (7, 8).

The remarkable survival of D. radiodurans cells to ionizing radiation and desiccation, which cause extensive DNA damage, has been rationalized under the hypothesis that protective mechanisms based on small-molecule Mn$^{2+}$ antioxidants preserve the high efficiency of DNA repair and replication proteins during irradiation (2, 9, 10). The dominant form of Mn accumulated in D. radiodurans cells is Mn$^{2+}$, with no significant levels detected of Mn$^{3+}$ or Mn$^{4+}$ before or after exposure to 10 kGy (1, 9), and the accumulation of high concentrations of Mn$^{2+}$ together with orthophosphate (Pi) and various organic metabolites is viewed as providing the components of “Mn antioxidants” that combat oxidative stress (11–13). When combined in vitro at physiological concentrations, these constituents interact synergistically, forming heat (100 °C)-resistance ROS-scavenging complexes that were extremely radioprotective of proteins, but not DNA or RNA (13, 15). As the antioxidant enzymes in D. radiodurans and other Mn-accumulating radiation-resistant prokaryotes are dispensable for radiation resistance (1, 6, 11), evidently, low-molecular-weight (LMW) Mn$^{2+}$–metabolite complexes, which make up ~70% of the cytosolic Mn of D. radiodurans (13), can provide levels of in vivo protection from ROS that equal or exceed the ROS-scavenging capacities of antioxidant enzymes (11).

The nature of the antioxidant Mn$^{2+}$ complexes within intact, viable D. radiodurans cells, and their response to radiation, has remained speculative, as there has been no means of determining Mn speciation in vivo; in particular, standard analytical procedures that disrupt cells alter speciation because Mn$^{2+}$ complexes rapidly exchange their ligands (13), and the idea that such Mn$^{2+}$ complexes can supplant ROS-scavenging enzymes remains controversial (2, 11, 16). We recently introduced (17) the use of advanced paramagnetic resonance techniques (18), electron spin-echo (ESE), electron paramagnetic resonance (EPR), electron nuclear double resonance (ENDOR), and ESE envelope modulation (ESEEM) spectroscopies, as a powerful, nondestructive way to study the in vivo coordination and speciation of Mn$^{2+}$ in viable cells, examining the possible role of Mn$^{2+}$-Pi as a key in vivo antioxidant in the simple eukaryote yeast, Saccharomyces cerevisiae (17). In this report, comparative measurements by these techniques reveal details of the in vivo Mn$^{2+}$ speciation (17) in D. radiodurans and E. coli cells, and of the responses of speciation when the cells are exposed to 10 kGy γ-irradiation. The results provide evidence that radiation protection in Deinococcus cells is indeed mainly mediated by Mn$^{2+}$-Pi complexes with metabolites, and give insights into the in vivo composition of the antioxidant complexes and their response to high doses of radiation. We further address a recent publication (16) that employs the spectroscopic
approach we introduced, but questions the central role of the LMW Mn$^{4+}$ complexes as radioprotectants.

**Results**

**EPR.** The EPR absorption spectrum of a Mn$^{4+}$ ion (S = 5/2) in high field is dominated by a feature centered at g-value of 2 (g-2) that is associated with transitions within the ms (±1/2) electron-spin manifold, as split into six lines by hyperfine interactions with the $^{55}$Mn (I = 5/2) nucleus. This feature is flanked by signals from "satellite" transitions involving the other electron spin substates (ms = ±3/2, ±5/2). The overall breadth of the pattern is proportional to the zero-field splitting (ZFS) parameter, D, whose magnitude in turn increases with deviations of the coordination sphere of the Mn$^{4+}$ ion from spherical symmetry (19). Figure 1 shows the 35 GHz ESE EPR (2K) spectra of Mn$^{4+}$ in frozen cell suspensions of *D. radiodurans* and *E. coli*, along with reference spectra of Mn$^{4+}$-Pi (17) and of the Mn$^{4+}$ fosfomycin-resistance enzyme, (FosA) (20). We first discuss spectra of LMW Mn$^{4+}$ complexes/standards (Fig. 1 and Fig. S1), then spectra of Mn$^{4+}$ enzymes (Fig. 1 and Fig. S2), and in this context then discuss the spectra of the cells (Fig. 1).

The spectra of hexaquo-Mn$^{2+}$ and Mn$^{4+}$-Pi are indistinguishable (Fig. S1), with a six-line $^{55}$Mn (I = 5/2) pattern centered at g-2 that "rides" on the overall g-2 feature that contains the response from the ms = ±1/2 electron-spin manifold plus contributions from the satellite transitions; an inflection on either side of the g-2 peak leads to the broadest satellite transitions, whose intensity vanishes below ~10 kG. The aquo and phosphate complexes have a highly symmetric (octahedral) coordination geometry, and the small spread of the satellite intensity corresponds to a small ZFS (D < 0.03 cm$^{-1}$). The ESE–EPR spectrum of Mn$^{4+}$ in a solution of N-methylimidazole (N-MeIm) (Fig. S1), chosen as an intensity standard for ESEEM measurements (21) of N-coordination, exhibits a slightly broader overall envelope than that of *D. radiodurans* and Mn$^{4+}$–Pi, and a less pronounced six-line $^{55}$Mn hyperfine pattern, with slightly lower $^{55}$Mn hyperfine coupling than Mn$^{4+}$–Pi. As confirmed by ESEEM measurements below, these effects are attributable to an increase in ZFS associated with the replacement of multiple (but fewer than six overall) water oxygens of hexaquo Mn$^{4+}$ by nitrogens of N-MeIm, resulting in a suite of complexes of slightly reduced symmetry at Mn$^{4+}$.

Mn$^{4+}$ in macromolecular environments can show small ZFS, for example Mn$^{4+}$ in Con A (D < 0.03 cm$^{-1}$) (22), but commonly shows lower symmetry and much larger ZFS (23, 24), leading to more pronounced wings associated with the satellite transitions. This is illustrated by the sample enzyme spectra in Fig. 1 and Fig. S2: as the ZFS parameter increases, the wings progressively broaden, with an accompanying broadening and splitting of the g-2 feature and loss of the six-line $^{55}$Mn hyperfine pattern: FosA (|D| ≃ 0.1 cm$^{-1}$) < FosA with bound substrate (FosA-S; |D| ≤ 0.2 cm$^{-1}$) < Mn-dependent superoxide dismutase, SodA (|D| ≤ 0.3 cm$^{-1}$) (24). Of particular note, the resolved six-line $^{55}$Mn pattern is already completely lost by D < 0.1 cm$^{-1}$ (FosA spectrum; Fig. S2, Inset). By comparison with the spectra of reference complexes and enzymes (Figs. S1 and S2), one can estimate an average of |D|~0.06–0.07 cm$^{-1}$ for the Mn$^{4+}$ in the N-MeIm solution.

The ESE–EPR spectrum of *E. coli* is distinctly different from that of hexaquo–Mn$^{2+}$ and phosphate-bound Mn$^{4+}$ (Fig. 1 and Fig. S1). The signal from the majority of Mn$^{4+}$ in *E. coli* instead corresponds to that of the low-symmetry Mn$^{4+}$ binding sites of enzymes such as SodA in having broad wings from auxiliary transitions whose intensity on the low-field side smoothly spreads from ~12 kG (g-2) down almost to zero (Fig. 1, Inset and Fig. S2); the smoothness of the broad wings likely reflects the superposition of signals from different enzymes with large but distinct ZFS parameters. Continuous wave (CW) and ESE–EPR spectra recorded at 9.7 GHz also are distinct from the standards (Fig. S3).

On the other hand, the *E. coli* spectrum also shows a well-resolved six-line $^{55}$Mn hyperfine pattern at g-2, even though this pattern is suppressed in all of the spectra of enzymes with Mn$^{4+}$ sites where D ≥ 0.1 cm$^{-1}$ (Fig. S2). We interpret this superposition of sharp lines on the broad majority signal to indicate the presence of a minority population of high-symmetry Mn$^{4+}$ ions with small ZFS, presumably the LMW complexes found in *E. coli* cells (13).

The ESE–EPR spectrum of late log-phase *D. radiodurans* is unlike that of *E. coli* or the enzymes, and quite similar to that of hexaquo–Mn$^{4+}$ and phosphate-bound Mn$^{4+}$ (Fig. 1 and Fig. S1) demonstrating that the *D. radiodurans* spectrum arises from Mn$^{4+}$ with a highly symmetric coordination sphere; the majority of this signal must be associated with the ~70% Mn$^{4+}$ LMW complexes (13). Closer inspection of the *D. radiodurans* Mn$^{4+}$ spectrum further reveals slightly broader satellite features that may be associated with LMW complexes of slightly lower symmetry or with the 30% of the Mn$^{4+}$ associated with high-symmetry protein sites. The *D. radiodurans* spectrum is essentially identical to those obtained previously for intracellular Mn$^{4+}$ of *S. cerevisiae* variants (Fig. S2) (17).

Most importantly, an expanded view of the low-field region of the ESE–EPR spectra of *D. radiodurans, E. coli,* and SodA (Fig. 1, Inset) shows that *D. radiodurans* exhibits no detectable intensity from the broad satellite transitions of low-symmetry Mn$^{4+}$ bound to SodA. Whereas the majority of the Mn$^{4+}$ of *E. coli* gives a spectrum characteristic of low-symmetry enzyme sites such as that of SodA, the absence of such a signal for *D. radiodurans* requires that no more than a few percent of total in vivo Mn$^{4+}$ in *D. radiodurans* can be present as SodA. This is so even though the SodA polypeptide (DR1279) is constitutively expressed in *D. radiodurans* grown under the conditions used here (Materials and Methods) (25). In short, although *D. radiodurans* has accumulated high concentrations of Mn$^{4+}$, and must contain the SodA polypeptide, the bacterium is seen to contain negligible quantities of the functioning Mn$^{4+}$-bound holo-enzyme.

These observations are broadly consistent with previous studies that showed that substantially more Mn is accumulated by *D. radiodurans* (0.2–2 mM Mn) than by *E. coli* (~0.01 mM Mn), with the majority of the "surplus" Mn$^{4+}$ (i.e., the portion of a cell’s Mn$^{4+}$ budget not bound to proteins) likely forming LMW ROS-scavenging complexes with various metabolites (1, 9, 13).

**Cellular $^{31}$P and $^{1}H$ ENDOR Spectroscopy.** ENDOR and ESEEM spectroscopies of a paramagnetic metal ion center such as Mn$^{4+}$ provide nuclear magnetic resonance (NMR) spectra of the nuclei that are hyperfine-coupled to the electron spin, and thus can be
used to identify and characterize coordinating ligands. Fig. 2 shows the 35 GHz Davies ENDOR spectra for the bacteria, *D. radiodurans* and *E. coli*. The frequency region from 17 to 37 MHz contains the response from the $^{31}\text{P}$ of phosphates bound to Mn, and that from 40 to 80 MHz from the $^{31}\text{P}$ of bound water (17, 26).

The ENDOR spectra are normalized to the ESE signal, allowing comparison of intensities for the two bacteria; numerical values for intensities of selected sharp features, denoted as $^{31}\text{P}^\%$ and $^{\text{H}}\text{H}^\%$ (17), are listed in Table S1. The frequencies of the $^1\text{H}$ and $^{31}\text{P}$ features accurately correspond to those seen previously for intracellular Mn$^{2+}$ of the *S. cerevisiae* variants (17).

The $^{31}\text{P}$ patterns for both bacteria include two sharp lines centered around the $^{31}\text{P}$ Larmor frequency separated by ~4.5 MHz. These lines are from the $m_s = \pm 1/2$ spin manifold of Mn$^{2+}$, and simulations show that they are associated with $^{31}\text{P}$ whose hyperfine coupling is almost isotropic, with isotropic coupling $A_{\text{iso}}$ simply distinguishing between Pi and pP or nucleotide binding.

*D. radiodurans* similar to those of the aquo and Pi standards (17), whereas the $\text{Mn}^{2+}$ ENDOR responses for *D. radiodurans* and *E. coli* are quite similar, which indicates that Mn$^{2+}$ complexes in the two bacteria have similar average fractional populations of bound phosphates. However, the $^{1}\text{H}$% values are unequal, *D. radiodurans* > *E. coli*, providing direct evidence for differences in the average occupancy of bound H$_2$O in the Mn$^{2+}$ coordination sphere for the two organisms. A heuristic quantitation of these observations within a model for speciation is given below.

**Cellular $^{14}\text{N}$ ENDOR Spectroscopy**

To quantitate $^{14}\text{N}$ ESEEM responses from Mn$^{2+}$ in the bacteria, we chose as a standard the $^{14}\text{N}$ response from bound N-MeIm. Fig. 3 shows the X-band three-pulse ESEEM time domain traces (Left) for *D. radiodurans* and *E. coli*, plus that for Mn$^{2+}$ plus N-MeIm, and the ESEEM spectra (Right) obtained by the Fourier transform (FT) of the corresponding time domain traces. The time domain data of the Mn$^{2+}$/N-MeIm standard shows strong $^{14}\text{N}$ ($I = 1$) modulation. The corresponding $^1\text{H}_2\text{O}$ ENDOR response from the Mn$^{2+}$/N-MeIm sample is substantially suppressed, indicating that multiple N-Me-Im bind to each Mn$^{2+}$ [the weakness of imidazole binding by Mn(II)] and the fact that Mn$^{2+}$/N-MeIm has an extremely small ZFS (32) suggests that in the N-MeIm standard solution, on average each Mn$^{2+}$ binds $\sim$3–4 N-MeIm, displacing waters (Fig. S4).

The associated ESEEM spectrum (Fig. 3, Right) exhibits a strong peak at $\sim$5 MHz, which is assigned as a double-quantum transition from $^{14}\text{N}$ of N-MeIm directly coordinated to Mn$^{2+}$. This frequency yields a $^{14}\text{N}$ hyperfine coupling of $A \sim$3 MHz as expected from single-crystal measurements on Mn$^{2+}$ (imidazole)$_6$ (32); additional weak low-frequency transitions seen in the frequency domain are primarily determined by quadrupole splittings, and a sharp feature near 2$\omega_a$ ~2 MHz can be assigned to a double-quantum transition from the remote $^{14}\text{N}$ of coordinated N-MeIm. Similar hyperfine couplings also are seen for Mn$^{2+}$ bound to nucleobases (33), and such a coupling thus may be taken as representative of nitrogenous ligands bound to high-spin Mn$^{2+}$.

The time domain trace for *D. radiodurans* shows $^{14}\text{N}$ modulation similar to that from the $^{14}\text{N}$ of N-MeIm (Fig. 3, Left), validating the use of this ligand as a standard. The modulation depth for *D. radiodurans* is $\sim$20% of the standard solution, which correlates to an average approaching one Mn$^{2+}$ ligand bound to each Mn$^{2+}$ complex of *D. radiodurans*; this interpretation is supported by the fact that the ESE–EPR spectra of *D. radiodurans* (Fig. 1) is not as broad as that of the N-MeIm standard (Fig. S1), in which Mn$^{2+}$ binds multiple $^{14}\text{N}$. The ESEEM spectra of *D. radiodurans* and Mn$^{2+}$/N-MeIm (Fig. 3, Right) both show features in the range of $\sim$2–6 MHz, in keeping with basic similarity of the time waves from the two samples. However, the differences in peak shapes and positions from those of N-MeIm, along with heavier damping of the $^{14}\text{N}$ responses, indicate the presence of multiple types of nitrogenous ligands.

The time wave for *E. coli* shows extremely weak $^{14}\text{N}$ modulation in the 0.5–1.0 $\mu$s range (Fig. 3, Left). Importantly, this modulation decays quickly, which suggests that it arises from a variety of species with different coupling constants and with low occupancy. Oscillations evident in the time domain traces are assigned to interference effects from $^{14}\text{N}$ ligands with different coupling constants. Given that the low-symmetry pool dominates the EPR spectrum and Mn$^{2+}$ binding to an enzyme invariably includes histidyl imidazole coordination, the extremely low modulation depth supports the conclusion that ENDOR/ ESEEM in *E. coli* also is dominated by the LMW complexes, and that these have extremely low occupancy of nitrogenous ligands.

The ESEEM spectrum for *E. coli* is weak (Fig. 3, Right), but in apparently extending to lower frequency than that from *D. radiodurans* is perhaps more similar to the N-MeIm spectrum than that of *D. radiodurans*. Presumably the $^{14}\text{N}$ spectrum from
SodA (and other enzymes) is broad, and therefore the modulation is weak and contributes little to the response.

**Cellular Mn\(^{2+}\) Speciation.** The absolute ENDOR responses for Mn\(^{2+}\) complexes, \(^{31}\)P\% and \(^{1}\)H\%, for *D. radiodurans* and *E. coli* (Table S1) can be given a heuristic interpretation in terms of the fractional populations, \(f_i\), for each of four classes of LMW species: Mn\(^{2+}\) complexes with bound (i) Pi, (ii) pP, and (iii) "ENDOR-silent" (ES) ligands, as well as (iv) the hexa-aquo-Mn\(^{2+}\) ion (denoted Mn\(^{2+}\)-aqua, Aq) (SI Text) (17). The ESEEM results presented above now show that ES ligands of *D. radiodurans* in fact include \(^{14}\)N-bound coordination sites as well as such truly "silent" ligands as the \(^{16}\)O of the carboxylates of metabolites and protein side chains, etc. In this context, we will simply refer to O/N ligands as ES, without making distinctions. As nitrogenous ligand(s) can bind to LMW Mn\(^{2+}\) along with Pi, the simple partitioning into four classes is no longer rigorous, but it remains heuristically useful as an aid in conceptualizing the significance of differences in \(^{1}\)H and \(^{31}\)P ENDOR intensities among cell types and genetic variants (17), in response to growth conditions (25, 34), or in response to \(\gamma\) radiation as in this report.

The heuristic Mn\(^{2+}\) speciation in *D. radiodurans/E. coli* is shown in Fig. 4A and Table S2. The \(^{31}\)P\% correspond to an in vivo fraction of \(\sim 30\%\) Mn\(^{2+}\)-bound Pi in both *D. radiodurans* and *E. coli*; neither has significant bound pP. As a result, the sum of the ES and hexaquo Mn\(^{2+}\) is essentially the same for the two organisms, \(f_{ES} + f_{Aq}\) \(\sim 70\%\). Fig. 4A shows that the two populations that make up this sum show striking but inverse relationships among the two bacteria. Thus, \(f_{ES} \sim 15\%\) for *D. radiodurans* is approximately half that of *E. coli* (30%), and \(f_{Aq}\) for *D. radiodurans* is approximately double that for *E. coli*.

The Mn\(^{2+}\) complexes in *D. radiodurans* not only show a strong \(^{14}\)N ESEEM response, but also a strong \(^{1}\)H ENDOR response (Fig. 2), which is reflected in the low value of \(f_{ES}\) and high \(f_{Aq}\) (Fig. 4A). At minimum, these results suggest that the 70% LMW Mn\(^{2+}\) of *D. radiodurans* retains many of the waters of aqua-Mn\(^{2+}\), in contrast, for example, to the Mn\(^{2+}\) in the high-concentration N-Melm solution or to the case of pP binding (17). For *E. coli*, \(f_{ES}\) is higher \((\sim 1/3)\), although little of the Mn\(^{2+}\) binds nitrogen, so the majority of the ES ligands likely are carboxylates.

**Changes Induced by \(\gamma\)-Radiation in Cellular Mn\(^{2+}\) Speciation.** \(\gamma\)-irradiation of *E. coli* significantly changes its ESE–EPR spectrum, broadening the auxiliary-transition “wings” (Fig. S1). Irradiation decreases the \(^{1}\)H% response for *E. coli* by \(-1/3\) while decreasing the \(^{31}\)P% only slightly (Fig. 4B), changes that imply a large \((-50\%)\) increase in the coordination of ES ligands by Mn\(^{2+}\) at the expense of the binding of water. Most dramatically, the \(^{14}\)N modulation for Mn\(^{2+}\) in *E. coli*, which is almost absent for unirradiated *E. coli*, increases sharply upon irradiation (Fig. S6), with the radiation-induced \(^{14}\)N ESEEM spectrum even exceeding in intensity that of *D. radiodurans* (Fig. 5), suggesting that the new ES ligands are nitrogenous species formed by radiation.

In sharp contrast, none of the spectroscopic probes of Mn\(^{2+}\) showed any change induced by 10 kGy in *D. radiodurans*; (i) there is no significant change in the ESE–EPR spectrum (Fig. S1); (ii) no change in water or Pi binding, as indicated by the invariant \(^{1}\)H% or \(^{31}\)P% ENDOR responses (Fig. 4B); and (iii)
none in $^{14}$N binding, as indicated by the invariant ESEEM response (Fig. 5).

These observations together indicate that $\gamma$-radiation–induced oxidative cleavage of proteins in E. coli generates nitrogenous products (peptides/amino acids) that bind through $^{15}$N to Mn$^{2+}$, replacing bound waters, whereas the proteins of D. radiodurans are protected.

**Discussion**

The accumulation of LMW Mn$^{2+}$ complexes as a critical mechanism of surviving $\gamma$-radiation independent of SodA has been established by the findings that: (i) SODs and catalases are dispensable in extremely radiation-resistant Mn-accumulating prokaryotes, without a significant loss in resistance when Mn is available (1, 2, 6–8, 11), a finding of such key importance that we reproduce representative earlier data in Fig. S7; (ii) limiting Mn accumulation renders wild-type D. radiodurans cells highly susceptible to radiation-induced protein oxidation and killing (1, 9); (iii) Mn-accumulating bacteria that lack SOD enzymes are able to dismutate radiation-induced O$_2^•$ to H$_2$O$_2$ at high efficiency (9); (iv) cell lysates of radiation-resistant prokaryotes contain a high abundance of heat-stable and dialyzable ROS-scavenging Mn-complexes that specifically protect proteins from $\gamma$-irradiation (6, 11, 13, 14); and (v) based on size exclusion chromatography of whole-cell, protease-inhibited aqueous extracts of D. radiodurans, relatively little Mn is bound to proteins (13). As Mn$^{2+}$ is innocuous under conditions where other biologically relevant redox-active metals (e.g., Fe) tend to promote ROS, cells can tolerate high cytoplasmic concentrations of Mn$^{2+}$ (1–30 mM), while complexes with Pi, peptides, and nucleosides scavenge O$_2^•$ during irradiation, preventing the proliferation of ROS in cells (9, 12, 13) and preserving the proteome’s functionality (2, 7, 10).

In this report, measurements by advanced paramagnetic resonance techniques build on these earlier findings, revealing differential details of the in vivo Mn$^{2+}$ speciation in D. radiodurans and E. coli cells, and of the responses of speciation to 10 kGy $\gamma$-irradiation. ESE–EPR shows that the Mn$^{2+}$ of D. radiodurans exists as LMW complexes, with negligible contribution from the Mn$^{2+}$ of holo-SodA (Fig. 1 and Fig. S2) even though the SodA polypeptide (DR1279) is constitutively expressed in D. radiodurans grown under the conditions used here (25). Perhaps the SodA in intact D. radiodurans cells is being out-competed for Mn$^{2+}$ by the accumulated LMW metabolites. Incomplete metallation of Mn-dependent enzymes has been reported for E. coli (4). Regardless, absence of ESE–EPR for SodA shows that the radiation resistance of D. radiodurans observed under these growth conditions (Fig. S7), and the invariance of Mn$^{2+}$ speciation to irradiation, seen by ENDOR/ESEEM, cannot be attributed to the action of SodA. It follows that E. coli, whose ESE–EPR signal is dominated by signals characteristic of SodA, but is without an adequate supply of “surplus” Mn$^{2+}$ in the form of LMW metabolite Mn$^{2+}$ complexes, is left highly susceptible to radiation-induced ROS and protein oxidation as previously reported (9, 13).

The in vivo $^{31}$P/$^4$H ENDOR and $^{14}$N ESEEM measurements show the LMW Mn$^{2+}$ complexes in D. radiodurans and E. coli have distinctly different speciation. In both organisms, these complexes show signals associated with coordination by phosphate and alanolamine. As expected (3, 11, 13, 17), however, the ESEEM spectra from the complexes of D. radiodurans show that in intact, unirradiated cells the LMW complexes incorporate substantial amounts of nitrogenuous metabolites, which give $^{14}$N signals whose intensities are inferred above to correspond roughly to one nitrogen ligand per LMW Mn$^{2+}$ complex. In contrast, the LMW complexes of unirradiated E. coli have extremely low occupancy of nitrogenuous ligands, with noticeably different characteristics. This disparity is consistent with (i) the demonstrated role of nitrogenuous metabolites in greatly enhancing the radioprotective properties of Mn$^{2+}$–Pi complexes (13, 15) and (ii) the 10–100 times lower concentrations of such N-containing metabolites in E. coli and other radiation-sensitive bacteria (13).

Exposure of D. radiodurans to 10 kGy causes no changes in the properties or speciation of the LMW Mn$^{2+}$ complexes, as an apparent corollary to the fact that the D. radiodurans cells are being protected from radiation damage mainly by these nonproteinaceous Mn antioxidants. In contrast, despite the presence of the holo-SodA in E. coli, the EPR spectrum of the Mn$^{2+}$ in E. coli is further broadened by 10 kGy radiation, and ESEEM shows that without an adequate supply of LMW Mn$^{2+}$ metabolite complexes in E. coli, the extensive protein oxidation caused by radiation-produced O$_2^•$ (and related ROS) yields radiation-induced nitrogenuous fragment species that bind Mn$^{2+}$. This interpretation is consistent with the observed proteome oxidation ($^{14}$N signals which in lethally irradiated E. coli (10, 13). Following exposure to 10 kGy, D. radiodurans displays no significant proteome oxidation, whereas cytosolic proteins in E. coli are highly oxidized by just 4 kGy (9, 10); protein fragmentation following 10 kGy correlates strongly with protein oxidation (13).

With these findings, we can briefly comment on a report that adopts the paramagnetic resonance approach we introduced (17) in a study of unirradiated D. radiodurans alone (16) but reaches the opposite conclusion to those included here. These authors argue that “it is this protein (SodA) and not smaller manganese complexes...that is probably the primary defense against superoxide.” In fact, their conclusion is contradicted by the studies described above (i–v), as extended here. In particular, we note the dispensability of SodA in radiation survival of D. radiodurans, Lactobacillus plantarum, and Halobacterium salinarum (as illustrated so powerfully in Fig. S7); the accumulation of an abundance of antioxidant metabolites in these extremely radiation-resistant prokaryotes (1, 6, 11), and of course, our current finding that in vivo Mn$^{2+}$ speciation in D. radiodurans is invariant to 10 kGy radiation when the D. radiodurans cells contain negligible amounts of holo-SodA. Indeed, under growth condition where D. radiodurans displays increased SodA activity, the cells are more sensitive to radiation (35), and the estimates in the recent report of SodA concentrations in D. radiodurans (16) are far higher than supported by the available literature (25, 35).

Returning to the present in vivo demonstration that nitrogenous metabolites are indeed components of antioxidant Mn complexes, to date a variety of N-containing metabolites accumulated in D. radiodurans have been identified as Mn$^{2+}$ partners in antioxidant defense when reconstituted in vitro, including peptides (5–20 amino acids in length), uridine, uracil, adenosine, inosine, and free amino acids (13), and the inorganic metabolite Pi (12, 13, 17). EPR/ENDOR/ESEEM studies of Mn$^{2+}$ bound to these candidates in vitro can help identify the functioning ligand(s) in vivo and reveal details of the cellular coordination environment of Mn$^{2+}$. As Mn$^{2+}$...
metabolites are becoming increasingly evident in all branches of life, and may also play an important role in microbial pathogenesis (11), the list of Mn-coordinating ligands that promote survival under oxidative stress is likely to expand. The advanced paramagnetic resonance techniques applied here will yield information about the structure and function of the Mn2+ complexes within living cells, and could further yield insights into how to better harness their radioprotective functions for practical purposes (2, 13, 15).

Materials and Methods

Sample Preparation. Strains, growth, and irradiation conditions: D. radiodurans (ATCC BAA-816) and E. coli (K-12) (MG1655) obtained from Michael Cashel (National Institutes of Health, Bethesda, MD). For spectroscopic analysis, strains were inoculated into 200 mL tryptone/glucose/yeast extract (TYG) growth medium (1) in 1 L flasks, and grown in an orbital shaker at 200 rpm to OD600 = 1 (late log-phase). D. radiodurans was grown at 32 °C. E. coli was grown at 37 °C. For each strain, 50 mL of culture were irradiated on ice (0 °C) to 10 kGy (3 kGy/h; 60Co, 10950±). Nonirradiated control cultures and irradiated cultures were harvested by centrifugation (4500g) as described (1). Nonirradiated control cultures and irradiated cultures were harvested by centrifugation (D. radiodurans, ~1018 cells; E. coli, ~1010 cells). The cells were washed twice with ultrapure water, resuspended in 0.5 mL 20% Glycerol (vol/vol)/ultrapure water, then frozen at ~80 °C.

Supporting Information

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**SI Text**

**Spectroscopic Speciation.** To calculate the % electron nuclear double resonance (ENDOR) response value (see Table S1), the measured $^{31}$P or $^1$H peak intensity (in volts) is scaled by the maximum electron spin-echo (ESE) intensity (volts) for that sample, measured using the two-pulse Hahn sequence, at the identical magnetic field that the ENDOR spectrum was collected. The ESE intensity was measured as the difference in the ESE maximum at that field to the response at zero applied field (the most reliable “off resonance field” because the EPR spectrum of Mn$^{2+}$ is so broad) (1).

**Heuristic Speciation Model.** As explained previously (1), the observed cellular ENDOR responses, $^{31}$P% and $^1$H%, can be given an heuristic interpretations in terms of the fractional populations, $f_i$, for each of four low-molecular-weight species present: manganous (Mn$^{2+}$) complexes with bound (i) P, (ii) pP, and (iii) ENDOR-silent ligands (denoted, Mn$^{2+}$-ES), as well as (iv) the hexa-aquo-Mn$^{2+}$ ion (denoted Mn$^{2+}$-aqua; Aq).

These estimates were based on the measured absolute ENDOR responses of the reference species, P, and H, obtained from Mn$^{2+}$ in aqueous solution, and the Mn(Pi)H2Ox and Mn(pP)2 complexes characterized through titration experiments.

$$^{31}\text{P}\% = P_P f_P + P_{pP} f_{pP}$$
$$^1\text{H}\% = H_{Aq} f_{Aq} + H_P f_P + H_{pP} f_{pP} + H_{ES} f_{ES}$$

$$f_P + f_{pP} + f_{ES} = 1$$

Of the four $f_i$, three (f$_{ES}$, f$_P$, and f$_{pP}$) are determined experimentally and the fourth, f$_{Aq}$ then is fixed by the normalization condition (Eq. S1.2). Of the three unknowns, f$_P$ and f$_{pP}$ can be expressed in terms of f$_{ES}$ and (parametrically) the Pi and pP concentrations through use of the corresponding phosphates binding isotherms, leading to the formulation of Eq. S1.1 in terms of the three unknowns, [Pi], [pP], and f$_{ES}$. It is possible to solve for the effective whole-cell f$_i$, namely the average whole-cell speciation, through use of the two experimental $^{31}$P% and $^1$H% ENDOR responses by assigning each cell line an effective Pi concentration equal to the measured cellular Pi concentration (Table S1); the results for the speciation for *Deinococcus radiodurans* and *Escherichia coli* are presented in Table S1.

Fig. S1. (Upper) Thirty-five gigahertz ESE–EPR spectra of the standards with 100 μM Mn²⁺. Conditions same as Fig. 1. (Lower) Thirty-five gigahertz ESE–EPR spectra of frozen cell suspensions of E. coli (Ec) and D. radiodurans (Dr). Before irradiation, black; after exposure to 10 kGy, red. Conditions same as Fig. 1.

Fig. S2. (Upper) Thirty-five gigahertz ESE–EPR spectra of superoxide dismutase (SodA), fosfomycin resistance protein (FosA), and FosA with the substrate fosfomycin (FosA-S) normalized to maximum intensity to compare shapes. (Lower) Spectra of E. coli (Ec), D. radiodurans (Dr), S. cerevisiae (Sc), and SodA normalized to unit area to highlight relative intensity. Fig. 1, Inset is the low-field portion of this figure. (Inset) Expanded g-2 regions. Conditions same as in Fig. 1. The EPR spectra for FosA and FosA-S are taken from ref. 1.

Fig. S3. X-band (Right) continuous wave (CW) EPR spectra and (Left) ESE–EPR of frozen solution of cells of *D. radiodurans* (*Dr*) and *E. coli* (*Ec*) before and after irradiation. Conditions: (Right) MW freq ~9.8 GHz; T, 4 K; mod amplitude, 4G. (Left) MW freq ~9.8 GHz; T, 10 K; tₚ, 16 ns; τ, 200 ns; tₑₛᴱ, 0.8 ms.

Fig. S4. Thirty-five gigahertz Davies ENDOR spectra of standard solutions of 2 mM Polyphosphate (pP), 1 mM N-methyl imidazole, 200 mM Pi, and hexa-aquo with just 100 μM Mn²⁺. Experimental conditions as in Fig. 2. Braces show the $^{31}$P, and $^1$H ENDOR response. $^{14}$N is the ENDOR response from N-Melm.
Fig. S5. (Upper) Thirty-five gigahertz 2 K Davies ENDOR spectrum of standard solution of 200 mM Pi with 100 μM Mn^{2+} and of *D. radiodurans* (Dr). Conditions as in Fig. 2. (Lower) Thirty-five gigahertz Mims ENDOR spectrum of standard solution of 200 mM Pi with 100 μM Mn^{2+}. Conditions: MW freq ∼34.8 GHz; T, 2K; ft, 50 ns; τ, 1,000 ns; t_{rep}, 20 ms; t_{rf}, 20 μs.

Fig. S6. Three-pulse electron spin echo envelope modulation time domain data at X-band for *D. radiodurans* (Dr) and *E. coli* (Ec) with and without irradiation (10 kGy). Unirradiated, 0; irradiated, 10. Conditions same as Fig. 3.
Fig. S7. Survival of strains exposed to acute γ-radiation (60Co). Solid square, *D. radiodurans* (wild-type, ATCC BAA-816) (pregrown in TGY, recovered on TGY); solid triangle, sodA− *D. radiodurans* (KKW7004) (1) (pregrown in TGY/8 μg mL−1 kanamycin, recovered on TGY/8 μg mL−1 kanamycin); solid diamond, *E. coli* (wild-type, MG1655) (pregrown in TGY, recovered on TGY). Growth, irradiation, and recovery conditions as described previously (2). Note, wild-type *D. radiodurans* (ATCC BAA-816) and sodA− *D. radiodurans* (KKW7004) are equally resistant to high-level chronic irradiation, displaying luxuriant growth on solid medium under 50 Gy/h (137Cs); *E. coli* (wild-type, MG1655) does not grow and is killed under 50 Gy/h (2). Values are from three independent trials with SDs shown.

Table S1. Absolute ENDOR intensities

<table>
<thead>
<tr>
<th>Organism</th>
<th>31P%</th>
<th>1H%</th>
</tr>
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<tbody>
<tr>
<td><em>D. radiodurans</em> 0 (Dr0)</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td><em>D. radiodurans</em> 10 (Dr10)</td>
<td>16</td>
<td>32</td>
</tr>
<tr>
<td><em>E. coli</em> 0 (Ec0)</td>
<td>16</td>
<td>25</td>
</tr>
<tr>
<td><em>E. coli</em> 10 (Ec10)</td>
<td>15</td>
<td>17</td>
</tr>
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</table>

See Spectroscopic Speciation in SI Text.

Table S2. Heuristic ENDOR derived average Mn2+ speciation

<table>
<thead>
<tr>
<th>Organism</th>
<th>31P%</th>
<th>1H%</th>
<th>[Pi]/mM</th>
<th>[pP]/mM</th>
<th>fPi(%)</th>
<th>fPp(%)</th>
<th>fAq(%)</th>
<th>fES(%)</th>
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<tbody>
<tr>
<td>Dr0</td>
<td>16</td>
<td>32</td>
<td>5</td>
<td>0</td>
<td>36</td>
<td>0</td>
<td>48</td>
<td>16</td>
</tr>
<tr>
<td>Dr10</td>
<td>16</td>
<td>32</td>
<td>5</td>
<td>0</td>
<td>36</td>
<td>0</td>
<td>48</td>
<td>16</td>
</tr>
<tr>
<td>Ec0</td>
<td>16</td>
<td>25</td>
<td>5</td>
<td>0</td>
<td>36</td>
<td>0</td>
<td>28</td>
<td>36</td>
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<tr>
<td>Ec10</td>
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<td>0</td>
<td>35</td>
<td>0</td>
<td>4</td>
<td>61</td>
</tr>
</tbody>
</table>


1. **ENDOR-response; 10x percentage change in ESE intensity.**
2. **Maximum [Pi] compatible with speciation model.**
3. **Fractions (f) of Mn2+ bound to Pi (i = Pi), pP (i = pP), ENDOR-silent ligands (i = ES), or present as Mn2+-aqua (i = Aq) calculated from [31P%, 1H%] (See SI Text).**
4. **D. radiodurans (Dr); E. coli (Ec); 0, 0kGy; 10, 10kGy.**