OPINION

A new perspective on radiation resistance based on *Deinococcus radiodurans*

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Abstract | In classical models of radiation toxicity, DNA is the molecule that is most affected by ionizing radiation (IR). However, recent data show that the amount of protein damage caused during irradiation of bacteria is better related to survival than to DNA damage. In this Opinion article, a new model is presented in which proteins are the most important target in the hierarchy of macromolecules affected by IR. A first line of defence against IR in extremely radiation-resistant bacteria might be the accumulation of manganese complexes, which can prevent the production of iron-dependent reactive oxygen species. This would allow an irradiated cell to protect sufficient enzymatic activity needed to repair DNA and survive.

One early goal of radiobiology was to explain why most organisms are so sensitive to X-rays and γ-rays. A whole-body exposure of just 10 Gy (gray; absorbed radiation dose) is lethal to most vertebrate animals, including humans[1], and most bacteria[1] cannot survive 200 Gy. Invertebrate animals are more resistant if the adult form has no somatic cell division (for example, fruit flies, which are largely post-mitotic)[2], and can survive 500 Gy. The freshwater invertebrate animal *Philodina roseola*[3], the water bear *Milnesium tardigradum*[4] and the roundworm *Caenorhabditis elegans*[5] can tolerate 3,000–5000 Gy, but are rendered sterile. As a haploid, the basidiomycete fungus *Ustilago maydis* carries a single set of chromosomes per cell in the G2 phase (after its chromosomes have been duplicated but before cell division) and can withstand ~3,600 Gy[6]. The archaeal species *Pyrococcus furiosus*[7] and *Halobacterium* sp. NRC-1 (REF. 8) can resist ~3,000 and ~5,000 Gy, respectively, whereas some bacteria from the *Deinococcus–Thermus* group can survive doses of more than 12,000 Gy[9]. Perhaps if we knew why some cells are so resistant to radiation, we could find ways to protect people from atomic radiation.

In the 1950s, DNA, lipids and proteins were proposed to be the main targets of ionizing radiation (IR) in cells. By the mid 1960s, 'death by DNA damage' became the predominant paradigm of radiation toxicity[10]. In the decades that followed, radiation biologists attempted to explain the extreme IR resistance of radioresistant organisms that can withstand extraordinary genome fragmentation and DNA base damage. This damage is caused by reactive oxygen species (ROS), the chemical agents that are principally responsible for cellular radiation damage[11] (BOX 1).

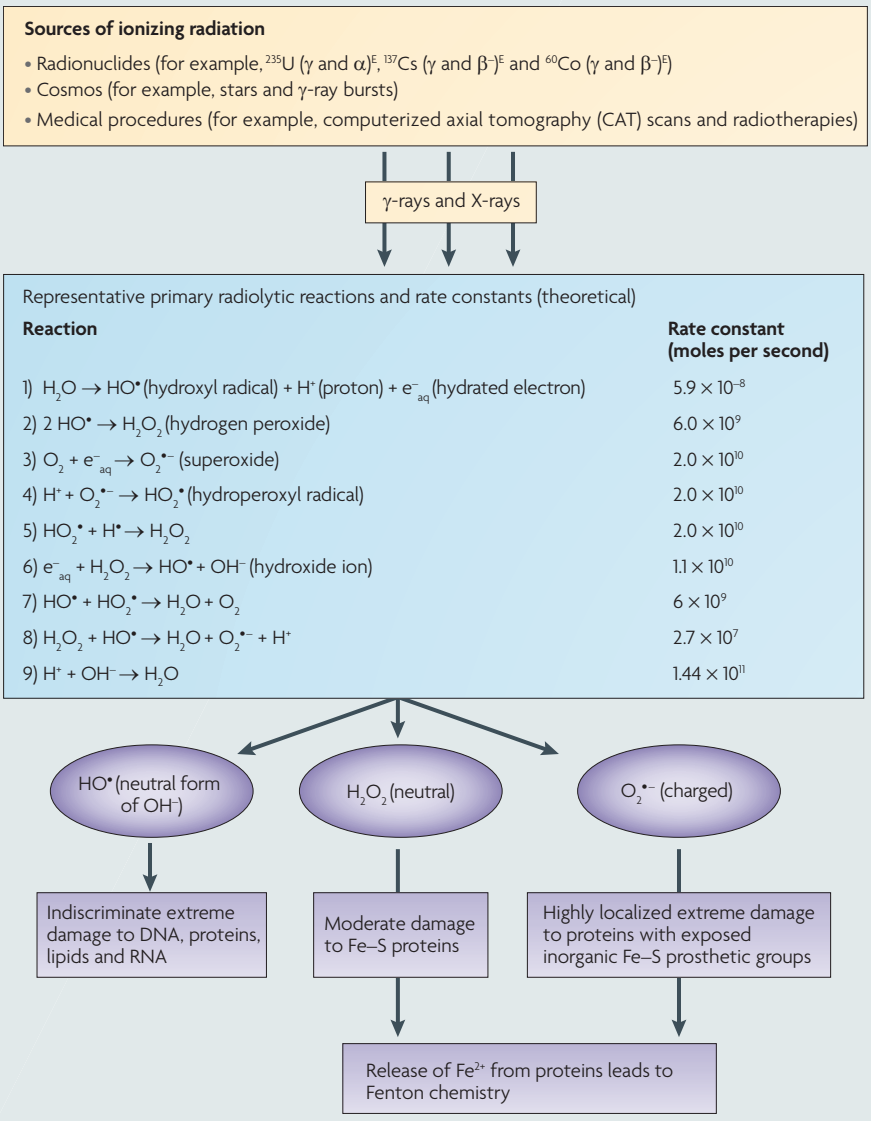
Because the fate of an irradiated cell ultimately depends on whether its genome is preserved, most studies have focused on how DNA that is damaged during irradiation is repaired[11–14]. DNA double-strand breaks (DSBs) are presumed to be the most lethal damage, although they are significantly less frequent than single-strand breaks (SSBs) and DNA base damages. IR damage that affects only one strand (for example, SSBs or damage to DNA bases) can be repaired using the undamaged complementary strand as a template, but DSBs provide little guidance for mending without mutagenesis, as neither of the two strands is intact. Non-mutagenic repair of DSBs is dependent on the presence of at least two sets of chromosomes per cell[12–15], although genome multiplicity by itself is insufficient for radioresistance. For example, *Escherichia coli* contains 4–8 haploid genomes per cell during exponential growth but cannot survive 200 Gy, which causes approximately 4 DSBs per haploid genome[14]. Because the linear density of genomic DSBs inflicted per Gy per Mbp (0.004–0.01) is similar for diverse organisms[3,9,12,16–18], during irradiation, cells with small genomes suffer fewer DSBs than cells with large genomes. For example, an acute dose of 1,000 Gy that inflicts ~30 DSBs in a 3 Mbp chromosome would be expected to inflict ~150 DSBs in a 15 Mbp chromosome. Therefore, cell survival would be predicted to decline as genome size increases, but significantly improve as ploidy increases to more than two. A similar result emerged for radiation-induced DNA base damage: the differences in base lesion yields for resistant and sensitive bacteria exposed to ultraviolet C (UVC) radiation (265 nm) or γ-rays were not nearly sufficient to account for the differences in bacterial survival[19,20].

UVC causes substantial direct damage to both DNA and proteins *in vivo*[19]. For proteins, UVC disrupts certain types of disulphide bonds and causes protein aggregation[21]. Antioxidants can increase the survival of cells exposed to UVC[21]. Thus, although cellular macromolecules are clearly not protected from direct damage caused by either UVC[19] or IR[9,16], any antioxidants in the cells would also be expected to help avert ROS-mediated toxicity that is secondary to the radiation itself; for example, caused by damaged proteins involved in energy metabolism[22]. Irradiated *Deinococcus radiodurans* is predicted to rid itself of damaged proteins using an expanded family of subtilisin-like proteases[23] that are expected to be highly expressed.

Radiation-resistant organisms suffer from similar levels of genomic damage following irradiation as sensitive organisms, but they can survive the formation of hundreds of IR-induced DSBs per genome[3,9,12,14,16–18]. The first reports of homologous recombination in an extremely IR-resistant organism

Box 1 | **Primary reactive oxygen species generated and their cellular targets**

H_2O is the most abundant chemical found in living cells. The primary source of reactive oxygen species (ROS) generated in irradiated cells is the radiolysis of H_2O, which generates $HO^•$, H^+ and e^-_{aq} (REFS 11,69) (see the figure, Equation 1). $HO^•$ radicals react with each other to generate H_2O_2 (REFS 11,69) (see the figure, Equation 2), which can diffuse throughout the cell[11,34]. Dissolved O_2, either derived from the atmosphere or generated endogenously, reacts with e^-_{aq} to form $O_2^{•-}$ (REFS 11,34,69) (see the figure, Equation 3), which can reform H_2O_2 in the presence of H^+ (REF. 69) (see the figure, Equations 4 and 5). Whereas H_2O_2 and $O_2^{•-}$ are relatively inert, $HO^•$ are extremely oxidizing, indiscriminately reacting with organic molecules and oxidizing DNA, RNA, lipids and proteins[11,34]. However, the short lifetime of the $HO^•$ precludes damage to molecules beyond a few Angstroms from where $HO^•$ is formed. For DNA, as the dose of ionizing radiation (IR) increases, the linear density of base damages and single strand breaks (SSBs) increases on both strands, which gives rise to double-strand breaks (DSBs). In addition to the widespread 'indirect damage' caused in cells by IR-induced ROS, IR also can inflict 'direct damage' when macromolecules absorb X-ray and γ-ray photons[11]. A dose of IR typically causes 40 times more SSBs than DSBs[9,11]. A secondary source of $HO^•$ in cells during irradiation is the Fenton reaction, which is one of the most powerful oxidizing reactions known and involves the catalytic decomposition of H_2O_2 by ferrous ions ($H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + HO^•$)[61] (FIG. 3); the analogous reaction with Mn(II) does not occur[51]. In contrast to $HO^•$, the reactivity of $O_2^{•-}$ is high only for selected targets: small inorganic prosthetic Fe–S groups of some proteins[57,61]. The damaging potential of $O_2^{•-}$ is greater than that of H_2O_2 for Fe–S groups because $O_2^{•-}$ is negatively charged.



were for *U. maydis*, which carries two sets of chromosomes per cell and can survive ~6,000 Gy as a diploid[6,13]. The only other extremely IR-resistant organism in which the physical products of homologous recombination during genome reassembly have been mapped following irradiation is the bacterium *D. radiodurans* (BOX 2), which as a polyploid (4–10 haploid genome copies per cell) can survive ~17,000 Gy[12,15,24] (FIG. 1a). *U. maydis* and *D. radiodurans* rely on efficient systems for genome reassembly and require the induction of DNA-repair genes[13,25]. Homologous recombination processes in these organisms depend on proteins that actively promote pairing of DNA, which results in efficient exchange of genetic information between homologous DNA regions[12–15]. Over the past two decades, several hypotheses to explain the efficiency of homologous recombination in *D. radiodurans* have been proposed, building on the idea that *D. radiodurans* maintains a novel chromosome alignment that keeps homologous regions together and facilitates access of broken ends to homologous templates[14,26,27]. However, a series of molecular studies on irradiated *D. radiodurans* cells showed that high levels of recombination between homologous DSB fragments originated from widely separated genomic locations[12,15,24,26], and cryoelectron microscopy of vitreous sections of *D. radiodurans* showed that DNA fragments in the cells were mobile[28]. Thus, IR-induced DSB fragments in *D. radiodurans* are not immobilized and the structural form of its nucleoids does not seem to have a key role in radioresistance[22]. Indeed, sensitivity to DSB damage can increase when the mobility of broken DNA ends is restricted[29].

Early on, it became evident that the enzymes which mediate DNA repair in *D. radiodurans* were probably not unique[30,31]. For example, a highly IR-sensitive mutant of *D. radiodurans* that contains a mutated DNA polymerase I gene (*polA*) was fully restored by expression of the corresponding gene from the IR-sensitive *E. coli*[30].

The nature of extreme IR resistance in *Deinococcus* spp. thus remains unclear and is made even more curious by their impressive ability to resist desiccation[14,16,32] and UV radiation[30,31]. Hypotheses that explain why repair proteins (either native or introduced by genetic engineering from IR-sensitive bacteria) in *D. radiodurans* cells function better after irradiation than repair proteins in other organisms generally fall into two categories. First, a subset of uncharacterized genes encode proteins that somehow facilitate the homologous recombination systems

of *D. radiodurans*[14,23,33], or second, the accumulation of near-millimolar concentrations of Mn^{2+} in cells somehow prevents protein oxidation during irradiation, with the result that sufficient repair enzymes survive radiation damage and allow subsequent DNA repair[16,22,26,34].

This Opinion article considers how, after exposure to huge doses of γ-rays, or to months of desiccation and exposure to UV sunlight in a desert, some bacteria retain sufficient protein activity to reconstitute their DNA. I propose that the accumulation of Mn^{2+} complexes in resistant organisms decreases the cellular concentration of ROS formed during irradiation. This in turn prevents IR-induced protein oxidation, which leaves sufficient recombination activity in place to repair the DSBs in the genome and enable survival.

The *Deinococcus* paradox

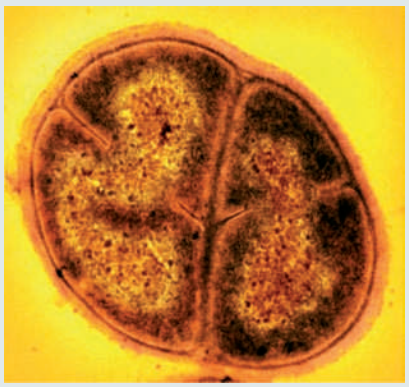
D. radiodurans was the first extremely IR-resistant organism to be sequenced[35]. Surprisingly, DNA-repair proteins in *D. radiodurans* seemed to be unremarkable, as the *D. radiodurans* genome encoded approximately the same number and types of DNA-repair proteins as IR-sensitive bacteria[23,26]. Similarly, the sequencing of genomes of the IR-resistant organisms *Bacillus pumilus*[36], *U. maydis*[6], *P. roseola*[3], *P. furiosus*[37] and *Halobacterium* sp. NRC-1 (REF. 38) did not provide clear insights into IR resistance mechanisms. Although the global transcriptional response to IR in *D. radiodurans* involved the upregulation of many novel genes, hundreds of characterized genes that were unrelated to DNA repair were also induced[25,39]. At least 12 novel *D. radiodurans* genes with discernible functional relevance to the preservation of genome integrity were knocked out, and the resulting mutants were characterized for IR resistance. Remarkably, there was no drastic change in the level of IR resistance of most of the mutants, indicating that few of the putative resistance genes, at least individually, make a substantial contribution to the recovery of irradiated *D. radiodurans*[26]. For example, DdrA is a protein that binds to and protects the 3′ ends of DNA from nuclease degradation *in vitro*, but 50% of *D. radiodurans ddrA*$^-$ mutant cells survived 10,000 Gy in an undefined rich medium[40]. Furthermore, a whole-genome analysis of gene gain and gene loss between *Deinococcus geothermalis* (BOX 2) and *D. radiodurans*, which are equally resistant to radiation[26], failed to produce a coherent picture of the systems that were thought to be important for survival. Instead, the number of novel genes that were thought to be implicated in recovery from IR in these *Deinococcus* species was substantially reduced[26].

Challenging radiation dogma

Because individual proteins are typically present at much higher levels than their corresponding genes, IR damage to one protein is not a lethal event, unlike an unrepaired DSB. As a dose of IR that causes indiscriminate cellular DNA damage should damage a similar portion of proteins in the same cell[11], the tacit assumption has been that IR-induced cell death is mainly mediated by DNA damage. However, recent investigations contradict this radiation dogma[34].

Whereas a specific dose of IR causes similar numbers of DSBs in sensitive and resistant bacteria[9,16,17], sensitive bacteria are more susceptible to oxidative protein damage than resistant bacteria[34] (FIG. 2). Based on this finding, I propose that the ability of IR-resistant organisms to minimize protein damage from IR allows them to retain sufficient DNA-repair functions to restore the chromosomal damage. I further argue that extremely IR-sensitive bacteria, such as *Shewanella oneidensis*, which are killed by IR doses (40 Gy) that cause less than 1 DSB per genome, do not survive mainly owing to protein damage[22,34,41]. Only for the few organisms that are extremely resistant to IR owing to their ability to protect proteins

Box 2 | **Where *Deinococcus* bacteria roam**



Representatives of the extremely radiation-resistant family Deinococcaceae can typically survive acute exposures to ionizing radiation (≥12,000 Gy (gray; absorbed radiation dose)), ultraviolet (≥1,000 J per m^2), and desiccation (years)[14,23,26,32,70], and can grow under harsh conditions of chronic irradiation (50 Gy per hour)[16]. Only approximately 30 distinct species have been described, despite their apparent ancient derivation[71]. The first member to be isolated was *Deinococcus radiodurans* (see the figure), originally named *Micrococcus radiodurans*[72]. This bacterium belongs to the *Deinococcus–Thermus* group, which is putatively related to cyanobacteria and is deeply branched in bacterial phylogenetic trees[23]. To date, the natural distribution of the deinococci has still not been explored systematically. Members have been isolated worldwide but have diverse and patchy distributions[23].

A wide range of natural and man-made environments on the Earth are characterized by their physical extremes of temperature, pressure and radiation. These harsh environments are different from the preferred environments of life typically encountered by humans, but can be colonized by *Deinococcus*. Some species live in highly radioactive soils at nuclear waste sites[73] and alpine environments[74], some have settled on sandstone, marble and ice in Antarctica[75], and others are ubiquitous microbial inhabitants of deserts[32]. High temperatures and pressures also do not seem to be an obstacle to their survival. *Deinococcus geothermalis* was originally isolated from a hot spring in Italy[76], and *D. geothermalis* DNA has been extracted from deep-ocean subsurface environments (68–118 meters below the sea floor)[77].

The astonishing survival of *Deinococcus* bacteria following irradiation has given rise to some whimsical descriptions of their derivation, including that they had an extraterrestrial origin[78]. Whole-genome comparisons of *D. geothermalis* with *D. radiodurans* and two *Thermus* species, however, have firmly established that the evolutionary steps which led to the *Deinococcus–Thermus* group occurred in their terrestrial ancestors[26]. *D. geothermalis* requires little more than some carbohydrates and oxygen to grow[79]. By contrast, *D. radiodurans* is metabolically less proficient. *D. radiodurans* requires a rich source of amino acids and vitamin B3 analogues in addition to sugars and oxygen for growth[79], representing biosynthetic deficiencies predicted by its genome sequence[23,35]. It is remarkable, therefore, that *D. radiodurans* has been isolated from nutrient-poor radioactive environmental waste sites and from deserts[32,73]. Indeed, numerous other *Deinococcus* species isolated from an elephant, a llama, a duck and a fish have similar growth requirements[23]. The picture that has emerged for the life cycle of most *Deinococcus* species is one that comprises a cell-replication phase that requires nutrient-rich conditions, such as in the gut of an animal[23], followed by release, drying and dispersal. Dried deinococci can endure for years, and if blown by winds through the atmosphere would be expected to survive and land worldwide — some becoming encased in ice, some becoming entombed in dried desert soils and some even ending up in the stomachs of humans[80].

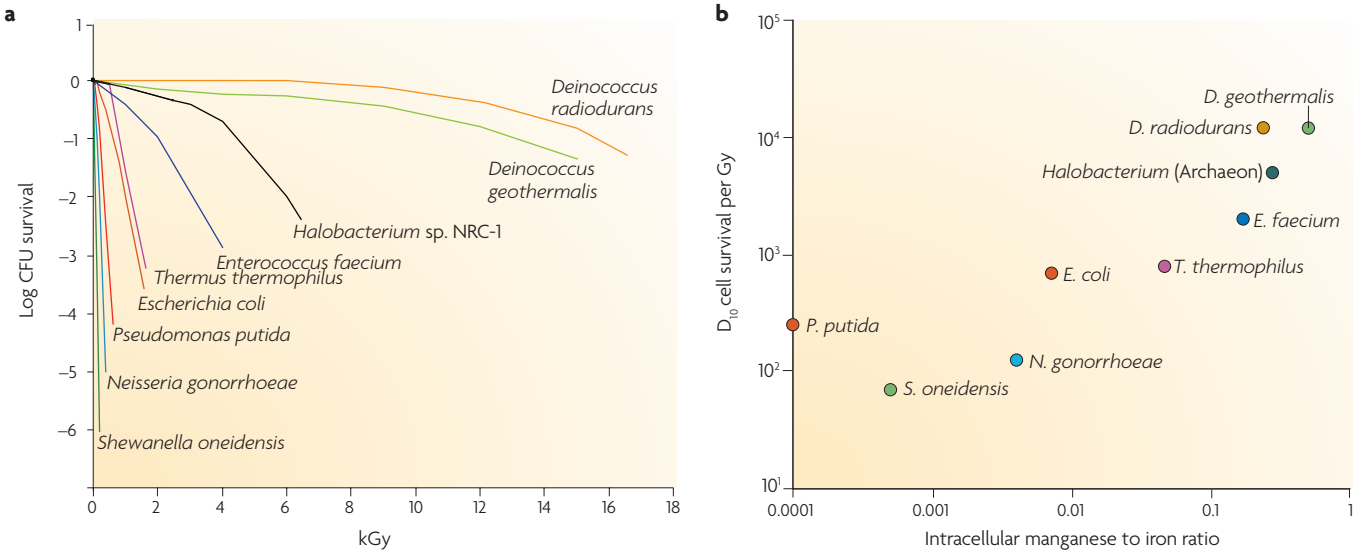


Figure 1 | **Relationship between survival following exposure to ionizing radiation and intracellular manganese and iron contents. a** | Ionizing radiation (IR) survival curves for whole-genome sequenced strains that encode a similar repertoire of DNA-repair proteins[8,16,38]. Standard growth, irradiation (^{60}Co at 0 °C) and recovery conditions were used[16]. **b** | Intracellular manganese to iron concentration ratios. For cells cultured under standard growth conditions, total manganese and iron contents were determined by inductively coupled plasma mass spectrometry[16]. D_{10} represents the IR dose that reduces the number of viable cells by 90%. Other manganese to iron ratios and D_{10} survival values reported recently for novel IR-resistant desert bacteria[32] include *Deinococcus radiodurans* 7b-1 (D_{10} = 13 kGy; manganese:iron = 0.14), *Deinococcus* sp. 1A1 (D_{10} = 17 kGy; manganese:iron = 0.15), *Deinococcus* sp. 5A5 (D_{10} = 15 kGy; manganese:iron = 0.38), *Deinococcus* sp. 1A6 (D_{10} = 7 kGy; manganese:iron = 0.15), *Deinococcus* sp. 3B1 (D_{10} = 10.5 kGy; manganese:iron = 0.12), alphaproteobacterium 4A4 (D_{10} = 1.5 kGy; manganese:iron = 0.07) and alphaproteobacterium 4A6 (D_{10} = 1.5 kGy; manganese:iron = 0.15). We did not investigate the distribution of manganese or iron in the desert strains, nor did we evaluate the extent of cell grouping before irradiation, which can significantly increase D_{10} survival values based on colony forming unit (CFU) assays[16,22]. Part **b** is modified, with permission, from REF. 81 © (2006) Elsevier Science.

from the indirect effects of IR (BOX 1) is survival ultimately determined by the level of DNA damage[12]. In the early 1970s, Holliday's work on IR-induced homologous recombination strongly supported the notion that the most radiosensitive targets in extremely IR-resistant *U. maydis* cells were single genes[13], an inference that has been applied by others to IR toxicity in general.

Resistance to DNA-damaging agents

In addition to IR and UV, *D. radiodurans* is profoundly resistant to the lethal and mutagenic effects of many redox-active (ROS-mediated) xenobiotics, such as mitomycin C[42], an antibiotic produced by *Streptomyces* that is known for its ability to cross-link DNA. Whereas *E. coli* is readily mutated by mitomycin C, *D. radiodurans* cells that survive mitomycin C treatment show approximately the same low level of mutagenesis that occurs during one normal round of replication[31,42]. Thus, *E. coli* has an error-prone DNA-repair pathway but *D. radiodurans* apparently does not[31]. Under our model, I attribute error-prone DNA repair to ROS-damaged enzymes, which passively promote mutations by repair malfunction[34]. By contrast, *D. radiodurans* and *E. coli* are equally sensitive to the mutagenic effects of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine, a direct mutagen[31,42] that was used to construct most *D. radiodurans* mutants prior to 1990 (REF. 31). Because DNA-repair-defective *D. radiodurans* mutants (such as *recA* and *polA* mutants) are approximately as sensitive to IR, UV and indirect (ROS-mediated) mutagens as repair-defective *E. coli*[9,30,31,43], accurate repair of DNA in *D. radiodurans* might require little more than protection against protein oxidation and genome multiplicity. I attribute protein protection in *D. radiodurans* to the presence of high levels of manganese complexes[16,34]. In radiation-resistant fungi and cyanobacteria, it is possible that resistance is conferred by different sorts of antioxidants, perhaps based on melanin[44] and trehalose[45], respectively.

Manganese within resistant bacteria

As no clear explanation has been inferred from the genome sequences of IR-resistant organisms for their survival capabilities, there have been no reliable physiological predictors of the ability of a cell to tolerate IR. In 1976, Bruce and colleagues reported that *D. radiodurans* accumulates substantially more manganese than the IR-sensitive bacterium *Micrococcus luteus*[46]. Furthermore, the IR-resistant bacterium *Lactobacillus plantarum*[16,47], which lacks the enzyme superoxide dismutase, and *Synechocystis* sp. PCC 68034 (REF. 48) accumulated exceptionally high levels of manganese[49,50]. The growth of IR-resistant bacteria was reported to be relatively independent of iron compared with IR-sensitive bacteria[16]. This set of reports led my research team to examine the relationship between manganese and iron accumulation and bacterial radioresistance[16]. FIGURE 1b shows the correlation between IR resistance and the ratio of intracellular manganese to iron concentrations for eight bacteria and one archaeon. For example, *D. radiodurans* (manganese:iron = 0.24) accumulates 157 times more manganese and 3.3 times less iron than the IR-sensitive *S. oneidensis* (manganese:iron = 0.0005)[16]. Bacteria with high manganese to iron ratios are extremely resistant to IR-induced protein oxidation, whereas bacteria with low manganese to iron ratios are hypersensitive to protein oxidation[16,32,34] (FIG. 2). Measurement of the accumulation of protein carbonyl groups[34] revealed that Mn^{2+} accumulation in bacteria specifically prevented protein oxidation, but did not affect DSB levels[16]. This led to the hypothesis that manganese-accumulating bacteria have an enhanced capacity to

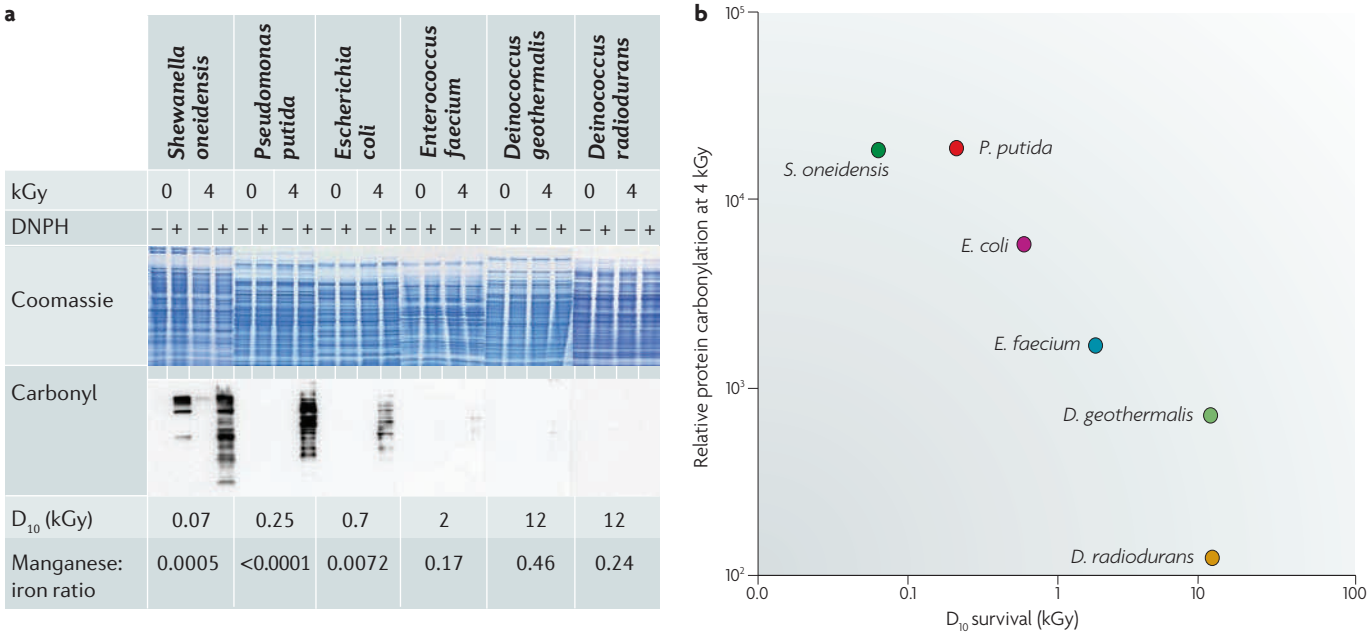


Figure 2 | ***In vivo* ionizing radiation-induced oxidative protein damage.** **a** | Western blot immunoassay of protein-bound carbonyl groups in cell extracts prepared from bacteria irradiated to 4 kGy (^{60}Co at 0 °C; 20 μg of a protein sample (soluble fraction) was loaded per lane). Coomassie represents a coomassie-stained polyacrylamide denaturing gel, whereas carbonyl represents the corresponding western blot, which reveals the presence (black) or absence of protein oxidation (no signal). Carbonyl groups (aldehydes and ketones) are widely used as markers of irreversible protein damage, which cannot be repaired and represents a small fraction of the total oxidative protein damage[34]. **b** | Relationship between bacterial survival (D_{10}; the ionizing radiation dose that reduces the number of viable cells by 90%) and relative protein carbonylation following exposure of the strains to 4 kGy. Carbonylation in relative units was quantified: the intensity profile of a particular lane (part **a**; carbonyl) was generated from previously published digitized membrane images[34]. DNPH, 2,4-dinitrophenylhydrazine. Part **a** is modified from REF. 34.

prevent the formation of iron-dependent ROS (through the Fenton reaction)[16,22,34] (FIG. 3). As the ability of manganese to protect the cell becomes depleted, DNA-repair systems and other essential cellular enzymes are expected to become increasingly subjected to damage[22,34]. This model explains the ability of orthologues from radiosensitive bacteria to complement *D. radiodurans* DNA-repair mutants, in which manganese complexes in *D. radiodurans* shield proteins irrespective of their origin[34]. It also explains the long 'shoulders' of IR-response curves for extremely resistant organisms (FIG. 1a), which are ultimately overwhelmed and killed owing to the hundreds of recalcitrant DSBs per cell[3,12] rather than protein damage[34].

Biological evidence

Direct evidence for a biological role of manganese accumulation in IR resistance comes from studies of *D. radiodurans*, which actively transports manganese into the cell[16]. Mn^{2+} sensing occurs widely in bacteria and influences both Mn^{2+} homeostasis and genes involved in the oxidative stress response[51]. *D. radiodurans* possesses two of the three types of known Mn^{2+} transporters: one from the Nramp (natural resistance-associated macrophage) family and one from the ATP-dependent ABC-type transporter family[16]. The third type of manganese transporter, the unique P-type ATPase with a high specificity for Mn^{2+}, has been detected in *L. plantarum*, but has not been found in *D. radiodurans*. Manganese transport in *D. radiodurans* is predicted to be regulated by a transcriptional regulator of the MntR–DtxR (manganese transport regulator–diphtheria toxin repressor) family by a metal-binding motif that is more closely related to the Mn^{2+}-binding configuration than the Fe^{2+} form[16,51]. Repression of manganese transport in *D. radiodurans* is predicted to be controlled by TroR[16].

When *D. radiodurans* was grown in conditions that limit manganese accumulation, the manganese to iron ratio of the cells decreased from 0.24 to 0.04, and the cells became highly sensitive to IR and highly susceptible to IR-induced protein oxidation, but there were no other obvious effects on other traits[16,34]. Furthermore, when *D. radiodurans* cells with normal manganese levels were irradiated under conditions that were known to perturb manganese-dependent ROS scavenging *in vitro*, such as high pH, the cells lost their ability to prevent IR-induced protein oxidation and became sensitive to IR[34]. Increasing evidence indicates that accumulated manganese ions can act as chemical antioxidant protectants by decreasing the levels of ROS to prevent oxidative stress. This mode of ROS control is not specific for radiation-resistant organisms, but is applicable to diverse settings. Addition of manganese can rescue yeast and bacterial mutants that are deficient in ROS-scavenging enzymes[51–53], and manganese supplementation can restore the lifespan of short-lived *C. elegans* mutants[54]. Mitochondria accumulate high Mn^{2+} concentrations[55], and the level of carbonylated proteins, which is recognized as a marker of oxidative stress, is reduced in animal cells[56] treated with Mn^{2+}.

Manganese and iron in IR resistance

The effects of radiation in cells are mediated primarily through ROS. Dissolved oxygen, either derived from the atmosphere, generated endogenously from IR-induced H_2O_2 by redox-cycling of iron, manganese and/or other biologically relevant transition metals, or produced enzymatically (for example, by the intracellular decomposition of H_2O_2 by catalase), reacts rapidly with electrons released during water radiolysis[11] to form superoxide ($O_2^{\bullet-}$) (BOX 1; FIG. 3). Because $O_2^{\bullet-}$ does not easily cross membranes, it can

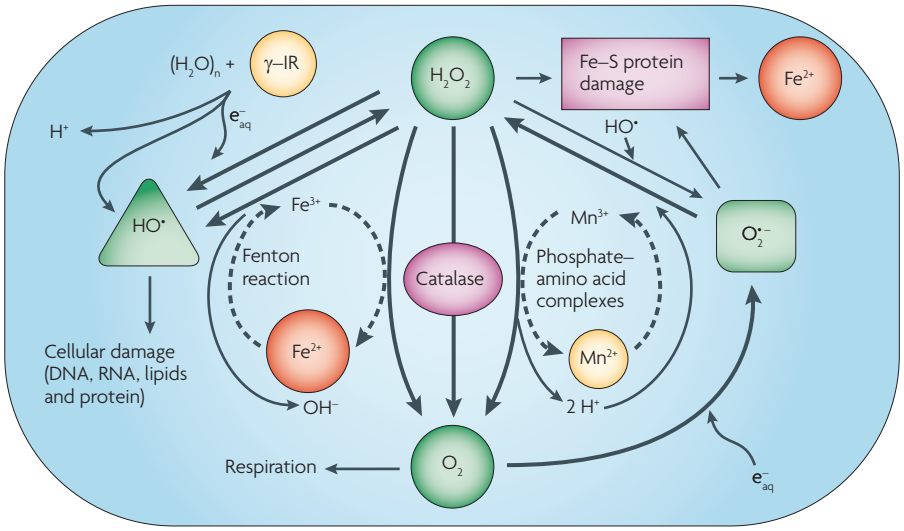


Figure 3 | **Model of ionizing radiation-driven manganese and iron redox cycling.** Under ionizing radiation (IR), Fe^{2+} and Fe^{3+} redox cycling is predicted to generate HO• and $O_2^{\bullet-}$, whereas Mn^{2+} and Mn^{3+} redox cycling is predicted to favour $O_2^{\bullet-}$ scavenging without HO• production. Catalase is a common enzyme that catalyses the decomposition of H_2O_2 to O_2 and H_2O. Thus, under anaerobic or aerobic irradiation conditions, in a cascade of events, $O_2^{\bullet-}$ would damage and inactivate enzymes with exposed Fe–S clusters[57,61], thereby triggering the release of Fe^{2+}, and 'free' Fe^{2+} would promote IR-driven Fe^{2+} and Fe^{3+} redox cycling. Fe–S clusters participate in a range of biochemical processes, including electron transfer, substrate binding and activation, redox catalysis, DNA replication and repair, regulation of gene expression, and tRNA modification. Thus, Mn^{2+} and Mn^{3+} redox cycling is predicted to prevent the proliferation of iron-dependent reactive oxygen species and protect diverse cellular functions[22,34]. Radiolysis of water exposed to ionizing radiation[11,69] (BOX 1), $H_2O + \gamma\text{-IR} \rightarrow HO^\bullet + H^+ + e^-_{aq}$; primary radiolytic reaction yielding H_2O_2 (REF. 11), $2\ HO^\bullet \rightarrow H_2O_2$; IR-induced superoxide[11], $O_2 + e^-_{aq} \rightarrow O_2^{\bullet-}$; Fenton reaction[61], $H_2O_2 + Fe^{2+} \rightarrow HO^\bullet + OH^- + Fe^{3+}$; Haber–Weiss reaction[11], $Fe^{3+} + HO_2^\bullet \rightarrow Fe^{2+} + O_2 + H^+$ and $2\ Fe^{3+} + H_2O_2 \rightarrow 2\ Fe^{2+} + O_2 + 2\ H^+$; manganese oxidation[34,49,51,58], $Mn^{2+} + O_2^{\bullet-} + 2\ H^+ \rightarrow H_2O_2 + Mn^{3+}$; manganese reduction[34,49,51], $2\ Mn^{3+} + H_2O_2 \rightarrow 2\ Mn^{2+} + O_2 + 2\ H^+$. Figure modified from REF. 34.

transiently build up in cells under aerobic or anaerobic irradiation conditions[34]. Although $O_2^{\bullet-}$ does not react with DNA or most proteins, it can damage and inactivate enzymes with exposed 2Fe–2S or 4Fe–4S clusters, which contain Fe^{2+} and can therefore trigger the release of ferrous ions (Fe^{2+})[57]. In turn, when Fe^{2+} ions are made accessible to H_2O_2 they can catalyse Fenton reactions[57] (FIG. 3). Thus, $O_2^{\bullet-}$ is not readily consumed by other cellular targets and the damaging potential of $O_2^{\bullet-}$ lingers for Fe–S clusters. I have proposed that Mn^{2+} ions that accumulate in resistant bacteria form complexes that shield Fe–S cluster-containing proteins from $O_2^{\bullet-}$-related ROS generated during irradiation (FIG. 3). By preventing the release of Fe^{2+} from Fe–S-cluster containing proteins, the global collateral damaging effects of Fenton chemistry during irradiation would be minimized, allowing enzyme systems involved in recovery to survive and function efficiently[22,34].

Because Mn^{2+} has a higher reduction potential than Fe^{2+} (REF. 51), Mn^{2+} is less likely than Fe^{2+} to donate an electron to reduce another molecule. Fe^{2+} thus catalyses the Fenton reaction, whereas the analogous reaction between Mn^{2+} and H_2O_2 does not occur[51]. All of the proposed benefits of manganese accumulation compared with iron accumulation in irradiated cells are related to the limitation of Fenton chemistry (FIG. 3). Like iron, manganese can cycle between the divalent and trivalent oxidation states[34,51]. In contrast to Fe^{2+} and Fe^{3+} redox cycling, Mn^{2+} and Mn^{3+} redox cycling during IR is predicted to favour $O_2^{\bullet-}$ scavenging without hydroxyl radical (HO•) production[34] (FIG. 3). Because the intrinsic reduction potentials of Mn^{2+} and Fe^{2+} are similar to those of many common biological compounds, each metal can be recruited, by carefully choosing the liganding environment, to perform biologically useful redox catalysis[51]. For example, as Mn^{2+} increases to millimolar concentrations, complex formation with ligands such as phosphate promotes catalytic $O_2^{\bullet-}$ scavenging during IR[34,58]. Indeed, Mn^{2+} efficiently scavenges for ROS at near-millimolar concentrations *in vitro*. Fridovich and Archibald demonstrated that Mn^{2+} scavenges $O_2^{\bullet-}$ in phosphate buffer[49], and Stadtman and colleagues detected Mn^{2+}-dependent disproportionation of H_2O_2 in bicarbonate buffer that contained amino acids[59], which endowed the mixtures with peroxidatic and catalatic activities[51]. Because Mn^{2+} is innocuous under conditions (notably aerobic environments) where Fe^{2+} tends to promote ROS, cells can tolerate high cytoplasmic concentrations of Mn^{2+} with virtually no negative redox consequences[51], unlike other biologically relevant redox-active metals. The IR-resistant bacteria *L. plantarum*, *Synechocystis* sp. PCC 6803 and *D. radiodurans* have intracellular Mn^{2+} concentrations that range from 1 to 30 mM[16,50,51,60]. Electronic paramagnetic resonance spectroscopy[16,46] and X-ray-absorption near-edge structure spectroscopy[34] revealed that manganese exists predominantly as divalent ions in *D. radiodurans*. An ample supply of Mn^{2+} would also ensure that the demands for this cation by manganese-dependent ROS-scavenging enzymes are met[61], and in cells in which iron is limited or sequestered, accumulated Mn^{2+} ions might functionally replace Fe^{2+} as mononuclear cofactors in enzymes, thereby protecting the active sites[61]. Thus, the accumulation of intracellular Mn^{2+} and its cofactors (for example, phosphate) might constitute chemical antioxidant protectants.

Location, location, location

The manganese-based protection hypothesis (FIG. 3) requires a global distribution of this metal within cells. X-ray fluorescence microspectroscopy showed that manganese is dispersed throughout *D. radiodurans* cells, but surprisingly, iron, as well as copper, which also catalyses the conversion of H_2O_2 to HO• *in vitro*, is sequestered between dividing cells (FIG. 4). By contrast, in *S. oneidensis* cells iron is dispersed throughout the cell[62]. Because of the sequestration of iron, the manganese to iron ratio in the cytosol of *D. radiodurans* is probably even higher than 0.24.

Banding patterns for oxidized proteins in carbonyl assays for sensitive bacteria exposed to IR[34] (FIG. 2a) revealed that some proteins are highly oxidized, whereas other proteins in the same cells are undamaged. By contrast, all proteins in *D. radiodurans* cells are resistant to IR-induced oxidation. However, proteins purified from *D. radiodurans* are not resistant to oxidation when irradiated *in vitro*[34]. As H_2O_2 generated during irradiation diffuses throughout a cell, any Fe^{2+} encountered (bound or unbound) would produce a torrent of ROS that would react with their immediate neighbours until either the H_2O_2 was exhausted or the iron was removed[57,61]. Owing to the short lifetime of HO• (BOX 1), HO• cannot damage

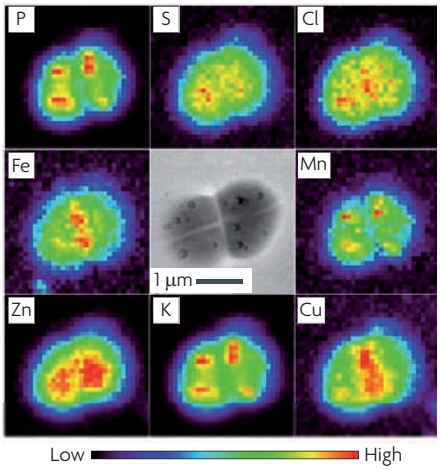


Figure 4 | **X-ray fluorescence maps of the qualitative distribution and concentration gradients of various metals and non-metals in *Deinococcus radiodurans*.** The tetracoccus was harvested from a mid-logarithmic culture in undefined rich medium, imaged and quantified as described previously[26,34]. The central panel is a transmission electron micrograph (TEM) of the tetracoccus after X-ray fluorescence (XRF) imaging. The panels that surround the TEM are the element distribution images of indicated metals and non-metals, which qualitatively depict high and low X-ray fluorescent intensities as indicated by the colour key (bottom): red represents the highest element concentration and black represents the lowest element concentration. A mathematical model of the original morphology of the cells was constructed in approximate likeness to the tetracoccus to determine the distribution of the elements[34]. XRF analysis measurements were made using the hard X-ray microprobe beamline 2ID-D at the Advanced Photon Source, Argonne National Laboratory, Chicago, USA, under previously described conditions[34,62]. Figure modified from REF. 34.

molecules beyond a few Angstroms from the ferrous ion where they are generated. One predicted repercussion is that proteins with predominantly negatively charged surfaces would become the focal points of Fenton chemistry during irradiation because they attract Fe^{2+}. This indicates that iron-dependent IR damage in sensitive cells is highly localized at the molecular level. By contrast, $HO^{\bullet}$ generated directly by the radiolysis of water would cause global, indiscriminate cellular damage (FIG. 3). The proposed perspective on IR toxicity has converged with a theory of eukaryotic ageing that postulates a causal relationship between oxidative protein damage and the decline of physiological functions and metabolic processes: carbonylation of specific proteins increases almost exponentially as animals age[63]. This model could also help us to understand the toxicity of redox-active heavy metals (for example, Cr^{6+} and Hg^{2+}) and hypochlorous acid (bleach), both of which would also be expected to knock down the activity of proteins they encounter in a selective manner[64].

This leaves the question of why chromosomal and plasmid DNA in extremely resistant bacteria are not similarly protected but instead are damaged with a similar dose dependence as DNA in sensitive bacteria[9]. Only 3% of a typical bacterial nucleoid by mass is DNA; the multitude of small proteins in the nucleoid that are involved in chromosomal condensation provide substantial shielding from $HO^{\bullet}$ generated by radiolysis. In the presence of cations (for example, Ca^{2+} and K^{+}), the genome is compacted by 1,000-fold by reducing the electrostatic repulsion between the negative charges of DNA[65,66]. When packaged in IR-resistant or IR-sensitive bacteria, DNA is more than 20 times as resistant to IR damage as when the same DNA is purified and irradiated in water[9]. Thus, DNA that is highly condensed in different organisms might be similarly susceptible to IR-induced DNA damage because of a combination of excluded water volume effects and the abundance of proteins[65,66]. In this context, DNA would remain prone to the direct damaging effects of IR, but might be out of reach of most of the indirect damage caused by $HO^{\bullet}$ generated by radiolysis (BOX 1). Provided that any iron atoms associated with DNA-binding proteins were tightly coordinated, and any Fe^{2+} or Fe^{3+} released within nucleoids was quickly removed and caged inside proteins, such as DPS (DNA protection during starvation)[67], DNA in bacterial nucleoids would be generally less prone to Fenton chemistry than regions of a cell where proteins with exposed Fe–S groups predominate.

Summary

Based on the radiochemistry of Mn^{2+} *in vivo* and *in vitro*[34,58], I propose that a combination of Mn^{2+} with ligands, such as phosphate[49,58], and perhaps other small molecules, spontaneously form intracellular complexes that act as potent catalytic scavengers[34,49,51,58,59] of $O_2^{\bullet-}$ and related ROS (FIG. 3). Mn^{2+} complexes are therefore expected to provide immediate cytosolic protection from IR-induced ROS. By shielding proteins with exposed Fe–S groups in particular, Mn^{2+} complexes would prevent the proliferation of iron-dependent ROS during irradiation in a way that is not highly dependent on the induction of enzymes, stage of growth or temperature over a range at which cells are metabolically active. It must be emphasized, however, that the fate of an irradiated cell in this 'death by protein damage' model rests not only on the level of protein oxidation, but also on the number of genome copies per cell and genome size. Cells that are proficient at homologous recombination but lack systems that can rejoin random DSB ends[15] (for example, non-homologous end joining) would not benefit from high levels of protein protection during irradiation if their genome copy number was less than two, as one DSB would be lethal. For polyploid cells, however, the presence of chemical antioxidant protectants of DNA-repair proteins would be expected to substantially increase the number of DSBs an organism could survive following irradiation.

A recent report shows that the dose-response relationship for desiccation killing in bacteria isolated from desert environments parallels the levels of protein damage and manganese to iron ratios[32]. Therefore, desiccation resistance also seems to depend on the abundance of cellular manganese complexes that protect proteins. The relative contribution to survival of accumulated Mn^{2+} ions compared with ROS-scavenging processes that have been classically attributed to enzymes has been explored for IR. Based on experimental analyses in *D. radiodurans*, Mn^{2+} accumulation trumps enzymatic ROS defence systems by far[16]; the genes that encode the constitutively and highly expressed ROS-scavenging enzymes *sodA* and *katA* can be disrupted in *D. radiodurans* with little effect on IR resistance, but the Mn^{2+} transporter gene (*nramp*) is essential[26]. The possibility that proteins with exposed Fe–S clusters are the most vulnerable macromolecules in irradiated cells (FIG. 3) warrants careful further investigation because of the practical implications. If the predictive power of IR-induced protein damage on bacterial survival extends to human cells exposed to low doses of IR, various analytical techniques could be applied to biodosimetry, which could be used to identify and quantify proteins that are covalently modified by ROS.

The ability of a complex eukaryotic genome to undergo extensive repair and rebuilding from the most extreme genomic insults is underscored by a recent report that diploid yeast cells can survive 250 IR-induced DSBs per cell[18]. This has led to the idea that higher eukaryotes could be made more resistant to IR. In 1968, Serianni and Bruce reported radioprotection of

E. coli (strain *B/r*) by a radioresistant factor extracted from *D. radiodurans*, but the nature of the protective agents and their cellular targets were not identified[68]. Based on insights presented in this Opinion article, delivery of *D. radiodurans* Mn^{2+} complexes into cells would be expected to protect proteins from oxidative damage and thereby increase survival. Several prominent questions now remain: can the putative antioxidant Mn^{2+} complexes of *D. radiodurans* be purified and identified and can they be used to prevent radiation injury and other forms of oxidative stress in human cells?

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DATABASES
Entrez Genome Project: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj
Bacillus pumilus | *Caenorhabditis elegans* | *Deinococcus geothermalis* | *Deinococcus radiodurans* | *Escherichia coli* | *Halobacterium* sp. NRC-1 | *Lactobacillus plantarum* | *Micrococcus luteus* | *Pyrococcus furiosus* | *Shewanella oneidensis* | *Synechocystis* sp. PCC 68034 | *Ustilago maydis*

FURTHER INFORMATION
Michael J. Daly's homepage: http://www.usuhs.mil/pat/deinococcus/index_20.htm

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OPINION

Antimicrobial peptides: linking partition, activity and high membrane-bound concentrations

Manuel N. Melo, Rafael Ferre and Miguel A. R. B. Castanho

Abstract | An increasing amount of information on the action of antimicrobial peptides (AMPs) at the molecular level has not yet been translated into a comprehensive understanding of effects in bacteria. Although some biophysical attributes of AMPs have been correlated with macroscopic features, the physiological relevance of other properties has not yet been addressed. Pertinent and surprising conclusions have therefore been left unstated. Strong membrane-binding and micromolar therapeutic concentrations of AMPs indicate that membrane-bound concentrations may be reached that are higher than intuitively expected, triggering disruptive effects on bacteria.

Antimicrobial peptides (AMPs) represent a wide range of short, cationic, gene-encoded peptide antibiotics that can be found in virtually every organism[1]. Different AMPs display different properties, and many peptides in this class are being intensively researched not only as antibiotics, but also as antivirals[2,3], templates for cell-penetrating peptides[4], immunomodulators[5] and antitumoural drugs[6].

Despite sharing a few common features (for example, cationicity, amphipathicity and short size), AMP sequences vary greatly, and at least four structural groups (α-helical, β-sheet, extended and looped) have been proposed to accommodate the diversity of the observed AMP conformations[7,8]. Likewise, several modes of action as antibiotics have been proposed, and there is debate about whether the primary target of many of these peptides is the cell membrane or whether the primary target is cytoplasmic invasion and disruption of core metabolic functions[9].

Several bilayer interaction and disruption models have been proposed for those AMPs that depend on membrane interference for their antimicrobial activity[10–12] (FIG. 1). However, it is now becoming obvious that such models might be too rigid to account fully for the many interactions that these small molecules can establish in a complex environment, such as the cell membrane. The limitations of the previously proposed models have been exposed in molecular dynamics simulations of AMPs interacting with phospholipid bilayers. Observations from these studies included multiple coexistent structures (frequently unrelated to clean α-helices or β-sheets), nonspecific peptide–peptide interactions and membrane perturbation dictated by stochastic events[13–15] (FIG. 1). The advantage of this indefinite behaviour is that bacteria seem to find it hard to circumvent AMP action, which is certainly a reason behind the multistep mutations usually required for resistance to evolve[16].

Independently of the chosen membrane perturbation model, an implicit concentration threshold is always required for disruption (FIG. 1). This is supported by several observations, in model systems, of phenomena related to such threshold crossings[13,17–19]. Nevertheless, AMP concentrations that are close to full membrane coverage, which are often considered unphysiological conditions, are a frequent requisite for such observations (TABLE 1).

In this Opinion, we survey reports of threshold events of AMPs and propose a correlation between these events and properties such as bactericidal concentration and membrane binding. We use this relationship to support our view, which reconciles existing interaction models with

> "Independently of the chosen membrane perturbation model, an implicit concentration threshold is always required for disruption."