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How radiation kills cells: Survival of *Deinococcus radiodurans* and *Shewanella oneidensis* under oxidative stress ☆

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Abstract

We have recently shown that *Deinococcus radiodurans* and other radiation resistant bacteria accumulate exceptionally high intracellular manganese and low iron levels. In comparison, the dissimilatory metal-reducing bacterium *Shewanella oneidensis* accumulates Fe but not Mn and is extremely sensitive to radiation. We have proposed that for Fe-rich, Mn-poor cells killed at radiation doses which cause very little DNA damage, cell death might be induced by the release of Fe(II) from proteins during irradiation, leading to additional cellular damage by Fe(II)-dependent oxidative stress. In contrast, Mn(II) ions concentrated in *D. radiodurans* might serve as antioxidants that reinforce enzymic systems which defend against oxidative stress during recovery. We extend our hypothesis here to include consideration of respiration, tricarboxylic acid cycle activity, peptide transport and metal reduction, which together with Mn(II) transport represent potential new targets to control recovery from radiation injury.
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1. What makes *Shewanella oneidensis* so sensitive to ionizing radiation?

The central dogma of radiation biology is that the cytotoxic and mutagenic effects of radiation are principally the result of DNA damage caused during the course of irradiation. This might not always be the case since environmental organisms such as *Shewanella oneidensis* (MR-1) (ATCC 700550) [1], which encode relatively complex DNA repair systems [2,3], are killed at radiation doses that cause relatively little DNA damage. The ionizing radiation doses that yield 17% survival of *Escherichia coli* and *Deinococcus radiodurans* are higher by factors of 20 and 200, respectively, than those for *S. oneidensis* [2]. Whereas 90% of *S. oneidensis* cells do not survive 70 Gy, a dose that induces less than one DNA double strand break (DSB) per genome, 10% of *D. radiodurans* cells survive 12,000 Gy, a dose that induces 120 DSBs per genome [2], and 10% of *E. coli* survive 700 Gy, a dose that induces 7 DSBs per genome (Table 1) [2]. Drying cells is also known to cause genomic DSBs [4], and *S. oneidensis* is killed after exposure to desiccation for only one day whereas similarly treated *D. radiodurans* can survive for months (Table 1) [2]. When the generation of reactive oxygen species (ROS) (superoxide, hydrogen peroxide and hydroxyl radicals) produced by irradiation or metabolism exceeds the capacity of endogenous scavengers to neutralize them, cells become vulnerable to damage, a condition referred to as oxidative stress (Fig. 1) [5,6].

Until recently, there have been no clear physiologic predictors of a cell's ability to recover from radiation or desiccation. In general, most of the resistant bacteria reported have been Gram-positive and the most sensitive have been Gram-negative [7,8]. However, there are several reported exceptions to this paradigm, the Gram-negative cyanobacterium *Chroococcidiopsis* is extremely radiation- and desiccation-resistant [9], whereas the Gram-positive *Micrococcus luteus* (*Sarcinia lutea*) is sensitive [10–12]. We recently reported that the differences in resistance to γ-radiation and desiccation for different bacteria mirror their intracellular Mn/Fe concentration ratios, where very high, moderate and very low Mn/Fe ratios correlate with very high, moderate and very low resistances, respectively [2]. *D. radiodurans* (Mn/Fe ratio: 0.24) accumulates 150 times more Mn than *S. oneidensis* (Mn/Fe ratio: 0.0005) and is sensitized to ionizing radiation when Mn(II) is restricted, and *S. oneidensis* accumulates 3.3 times more Fe than *D. radiodurans* (Table 1) [2]. In the case of *S. oneidensis* exposed to doses $\geqslant$70 Gy, Fe(II)-dependent oxidative stress produced

Table 1
Relationship between *c*-type cytochrome number, Mn/Fe levels and resistance

Strain (genomic sequence)	Genome size (Mb)	Total no. of *c*-type cytochromes	[a] Intra-cellular Mn/Fe ratio	[b] D_{10}, IR survival (kGy)	[b] D_{10}, desiccation survival (days)
[c]*Deinococcus radiodurans*	3.28	7	0.24	10–12	>30
[d]*Deinococcus geothermalis*	3.23	7	0.46	10	>30
[e]*Escherichia coli*	4.64	6	0.007	0.7	7
[f]*Pseudomonas putida*	6.18	17	<0.001	0.25	2
[g]*Shewanella oneidensis*	5.13	39	<0.001	0.07	1

[a] Mn/Fe ratios were previously reported [2].
[b] 10% cell-survival values (D_{10}) for ionizing radiation (Gy) or desiccation (days) were previously reported [2].
[c] http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = genomeprj & cmd = Retrieve & dopt = Overview & list_uids = 65.
[d] Draft annotation at Oak Ridge National Laboratory, Oak Ridge TN (http://genome.ornl.gov/microbial/dgeo/).
[e] http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = genomeprj & cmd = Retrieve & dopt = Overview & list_uids = 225.
[f] http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = genomeprj & cmd = Retrieve & dopt = Overview & list_uids = 267.
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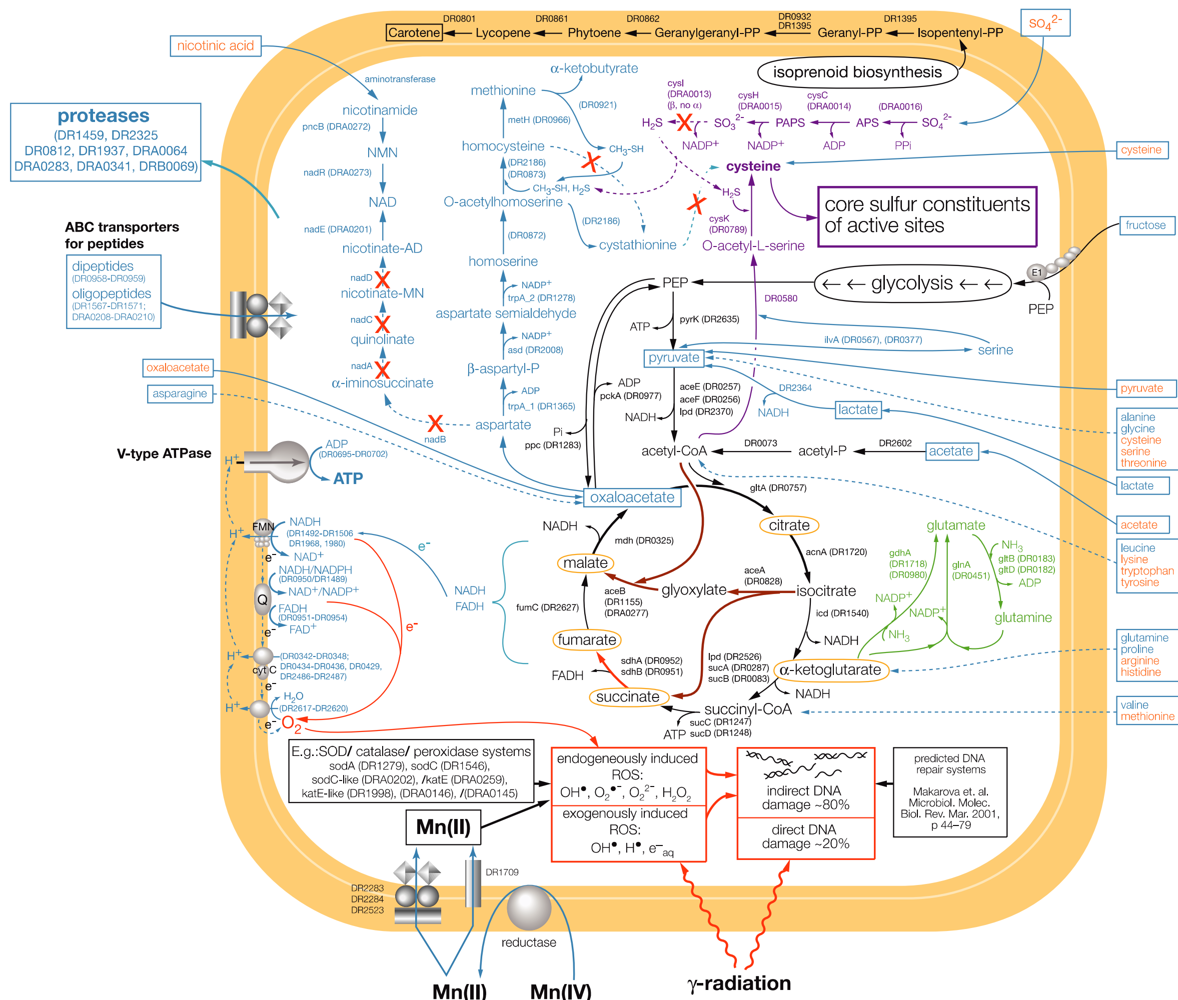

Fig. 1. Overview of the basic metabolic pathways of *D. radiodurans* and systems that generate and defend against ROS (reactive oxygen species). The pathways are color-coded as follows: Black, catabolism of fructose, lactate and acetate; pyruvate oxidative decarboxylation; TCA cycle; and carotenoid biosynthesis. Brown, reactions of the glyoxylate bypass. Blue, methionine, serine biosynthesis, and electron transfer coupled to ATP synthesis. Purple, cysteine biosynthesis and sulfate reduction. Green, basic ammonia metabolism. For each reaction, the gene name and/or *D. radiodurans* gene number is shown. Red crosses show the location of predicted disruptions (missing genes) of biosynthetic pathways. Red arrows and boxes show the dominant reactions leading to the generation of ROS. Black boxes include potential defense systems against free radical damage. Black box around Mn(II), accumulated non-enzymic Mn(II)-based scavengers of $O_2^{\bullet-}$ and related ROS. Blue boxes outside the cell show proteases and substrates: blue substrates in these boxes have predicted specified transporters, orange substrates do not have predicted specified transporters, but some are believed to be transported based on experimental evidence (Table 2). Long dashed blue lines show conventional pathways leading to degradation of amino acids. Long solid blue lines indicate transport based on experimental evidence (Table 2). Inside the cell, blue boxes indicate substrates that support moderate/poor growth, orange ovals indicate substrates that were tested and shown not to support growth (Table 2). Abbreviations: APS, adenylylsulfate; PAPS, phosphoadenylylsulfate; NAD, nicotinamide adenine dinucleotide (oxidized form); NADH, nicotinamide adenine dinucleotide (reduced form); NADP, nicotinamide adenine dinucleotide phosphate (oxidized form); NMN, nicotinamide mononucleotide; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide (oxidized form); FADH, flavin adenine dinucleotide (reduced form); ATP, adenosine triphosphate; ADP, adenosine diphosphate; PEP, phosphoenolpyruvate; nicotinate-AD, nicotinic acid adenine dinucleotide; nicotinate-NM, nicotinic acid mononucleotide; E1, subunit of phosphotransferase system; Pi, orthophosphate; PPi, pyrophosphate; Q, ubiquinone; SOD, superoxide dismutase; $O_2^{\bullet-}$, superoxide radical; O_2^-, peroxide ion; H_2O_2, hydrogen peroxide; $HO^\bullet$, hydroxyl radical; and e_{aq}^-, hydrated electrons.

during recovery might lead to additional DNA, RNA, lipid and protein damage [2].

2. Competing views of radiation resistance mechanisms

Studies have shown that for a given dose of ionizing radiation, the number of DNA double strand breaks in *D. radiodurans* compared to *S. oneidensis* is about the same, and that genomic DNA of *D. radiodurans* is not endowed with unusual protection from in vivo irradiation [2,13]. Thus, the key to surviving DNA damage appears to reside in the ability of *D. radiodurans* to repair DNA, and two competing views of the mechanisms responsible for its extraordinary survival now dominate [2]: (i) there are repair functions encoded among its

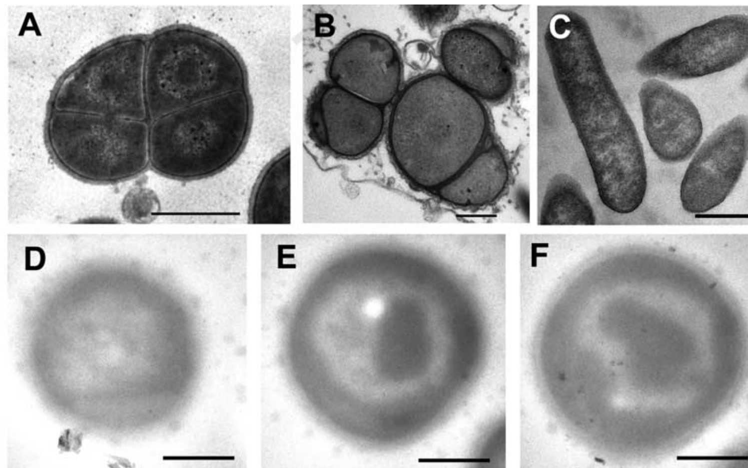

Fig. 2. Transmission electron microscopy. Unpublished images derived from work reported in Daly et al. [2]. (A) *D. radiodurans* (ATCC BAA-816), tetracoccus, grown in TGY (rich medium) (stationary-phase); condensed RN morphologies are visible. (B) *D. radiodurans* diplococci, grown in defined minimal medium (logarithmic-phase); diffuse genomes. (C) *S. oneidensis* (MR-1) grown in DSM (defined *Shewanella* medium [2]), stationary-phase. (D–F) Three serial micrographs of the same *D. radiodurans* cell, grown in TGY (stationary-phase), support that the nucleoid has a sphere/coralline-like structure similar to that reported in *E. coli* [19]. Scale bars, 0.5 μm. See Appendix for experimental details.

hypothetical genes, but most novel genes implicated in recovery by transcriptome profiling have had little effect on survival when disrupted [14]; or (ii) a combination of non-enzymic and enzymic antioxidant defenses allow relatively conventional repair pathways to function with greater efficiency than in other bacteria [2]. A third view, that highly condensed 'ringlike' nucleoids (RNs) are key to resistance [15] is generally discounted [2,16]. Specifically, Levin-Zaidman et al. [15] have proposed that each cell of a typical *D. radiodurans* cluster (tetracoccus, four cells) (Fig. 2A) contains a single copy of its genome partitioned as a ringlike nucleoid (RN), and following irradiation, is involved in a two-stage repair process. Where, in the first stage, DNA fragments arising from DNA double strand breaks are prevented from defusing by a ridged, toroid-shaped nuclear matrix and undergo error-free blunt-end joining; and in the second stage, RNs in adjacent cells fuse and undergo template-specific recombination. The overriding reasons why such a model is unlikely to be correct include: (i) each cell of tetracoccus contains 4–8 copies of its genome [17]; (ii) blunt-end joining of chromosomal DNA double strand breaks in *D. radiodurans* is not observed [18]; (iii) *D. radiodurans* cells grown to the logarithmic-phase in defined minimal medium do not display condensed RNs (Fig. 2B), but are equally resistant [2]; (iv) several other highly resistant deinococci do not form condensed RNs [2] (http://www.usuhs.mil/pat/deinococcus/index_20.htm); and (v) the relatively sensitive bacterium *E. coli*, which accumulates 18 times less intracellular Mn than *D. radiodurans* [2], displays RN nucleoid morphologies by transmission electron microscopy (TEM) [19,20] and fluorescent microscopy [21], but cross-sections support sphere/coralline-like structures with central cores of unknown composition [19], not toroids; sections through random planes of highly condensed late stationary-phase *D. radiodurans* nucleoids typically reveal circular-shaped structures [2,22] (Fig. 2). For *S. oneidensis* (Fig. 2C), we have proposed that ROS generated by energy-metabolism after irradiation during recovery might underlie this organism's great sensitivity [2]. One source of electrons which can leak from the substrate-side of the respiratory chain is heme-containing *c*-type cytochromes, yielding the reduced oxygen-product superoxide anion ($O_2^{\bullet-}$) (Fig. 1) [5]; $O_2^{\bullet-}$ can be converted to other ROS such as hydrogen peroxide (H_2O_2) by dismutation, and the extremely reactive hydroxyl radical ($HO^{\bullet}$) produced by Fe(II)-dependent reduction of H_2O_2 (Fenton-type chemistry) [5]. In this context, we note that *S. oneidensis* has one of the highest numbers of *c*-type cytochromes, as predicted from genome sequence, and iron contents reported for any bacteria (Table 1) [1,2].

3. Manganese-dependent bacteria

Whereas Mn(II) salts are soluble, Mn(III,IV) oxides are relatively insoluble at circaneutral pH, and both forms are widely distributed in the environment [23]. Manganese-dependent microorganisms such as *Deinococcus*, *Arthrobacter*, *Bacillus*, *Streptococcus* and cyanobacteria spp. have been implicated in the deposition of manganese oxides in dark manganiferric rock varnish coatings on desert rocks [24–26]. Organisms that belong to those groups are known for their radiation and desiccation resistance, and our recent work establishes a link between the role of Mn(II) and an environmental niche they occupy [2]. The existence of high intracellular Mn/Fe concentration ratios

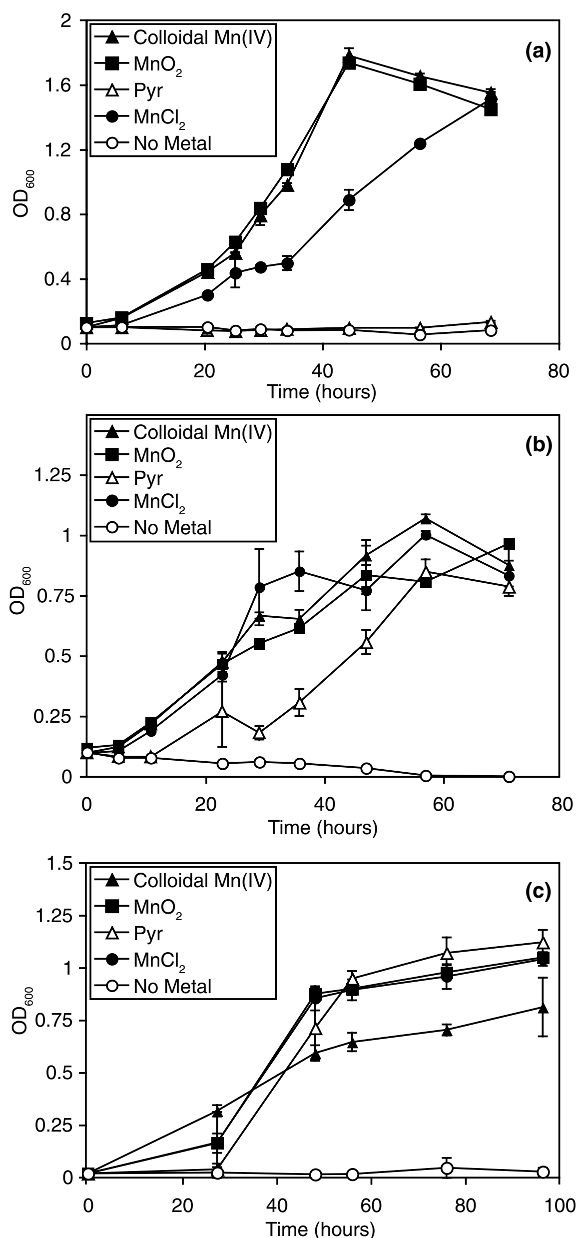

Fig. 3. Growth with Mn oxides. (a) *D. radiodurans* R1 (ATCC BAA-816), (b) *D. radiodurans* 7b-1 [30], and (c) *Deinococcus geothermalis* (DSM 11300) [62]. No metal, no Mn or Fe added. Mn sources: Colloidal Mn(IV) oxide, product of permanganate oxidation; Pyr, pyrolusite, β-MnO_2 (mineral); $MnCl_2$, manganous (Mn(II)) chloride; MnO_2, Mn(IV) oxide. See Appendix for experimental details.

in phylogenetically distant, radiation resistant bacteria but not in sensitive cells supports the idea that Mn(II) accumulation (with relatively low Fe) might be a widespread strategy that facilitates survival [2]. Accumulation of high intracellular levels of Mn(II) as a mechanism to fight $O_2^{\bullet -}$-related ROS has been reported for a variety of bacteria including *Lactobacillus*, *Borrelia*, *Neisseria*, *Deinococcus* and *Enterococcus* spp. [2,27–29].

Fig. 3 shows that *D. radiodurans* (R1 and 7b-1) [2,30] and *Deinococcus geothermalis* [31] are able to utilize colloidal Mn(IV) oxides for growth in defined minimal medium [2]. These organisms grew equally well when supplemented with Mn(IV) oxides in place of $MnCl_2$, suggesting that they possess the ability to reductively mobilize solid-phase Mn(IV), although abiotic reduction to Mn(II) by growth medium components can not be ruled out. We have previously reported metal reductase activities in these organisms [31,32], and it is possible that such functions might facilitate the reductive assimilation of environmental sources of Mn(III,IV) oxide. The mechanism by which high levels of intracellular Mn(II) scavenge $O_2^{\bullet -}$ and related ROS in the absence of superoxide dismutase (SOD) and catalase is not understood [33], nor is it known if Mn(II) acts as bound ions or as non-enzymic catalytic complexes. Reactions that might be relevant to the scavenging ability of intracellular Mn(II) include those which mimic activities of SOD: $\mathrm{Mn(II)} + O_2^{\bullet -} + 2H^+ \rightarrow \mathrm{Mn(III)} + H_2O_2$; oxidases: $\mathrm{Mn(II)} + 2O_2^{\bullet -} \rightarrow \mathrm{Mn(IV)}$ oxides; and deprotonation: $\mathrm{Mn(II)} + HCO_3^- + H_2O_2 \leftrightarrow HCO_3^-\text{–}\mathrm{Mn(II)}\text{–}{}^-OOH + H^+$ [33–35].

Nucleic acids coordinate Fe(II) very well [36]. However, even though Fe levels in *D. radiodurans* are four times higher than Mn [2], the chromosomes of *D. radiodurans* preferentially bind Mn(II) [2,10]. Mn(II) binds predominantly at the N7 position of guanine, possibly leading to preferential binding of Mn(II) over Fe(II) within DNA duplexes [36]. Whereas high intracellular Mn(II) concentrations do not protect DNA during in vivo ionizing radiation [2,13], Mn(II) ions can mimic as catalase- [35] and SOD-activities [33–35,37,38], and are not associated with the production of $HO^{\bullet}$ by Fenton-type chemistry in vivo [33]. In contrast, "free Fe(II)" meaning Fe(II) that is not incorporated into enzymes or iron-storage proteins catalyzes Fenton-type chemistry in vivo [5,6], and has been reported to be released from its bound state in cells by oxidative stress and radiation [39,40]. Since the production of $O_2^{\bullet -}$ is intimately associated with respiration, the case can be made that Mn-rich, Fe-poor cells are less susceptible to metabolism-induced ROS during recovery than Mn-poor, Fe-rich cells. After exposure to ultraviolet radiation (UV), desiccation or ionizing radiation, non-enzymic Mn(II)-based protection might facilitate survival by preventing sudden increases in metabolism-induced, Fe(II)-dependent ROS at the onset of recovery [2,41] and before enzymic defense systems are recalibrated [5]. Supporting this model, *D. radiodurans* cells are able to express very high levels of SOD and catalase [42,43], but are sensitized to ionizing radiation by limiting Mn(II) [2]. In contrast, *S. oneidensis*, which does not accumulate Mn(II), encodes a diverse set of protection systems [2] but is extremely sensitive to oxidizing conditions [2,44].

4. Role of metabolism in recovery of *D. radiodurans*

Our whole genome transcriptome analysis of *D. radiodurans* recovering from ionizing radiation (15 kGy) supports the idea that cells limit biosynthetic demands during recovery by importing exogenous peptides and other secondary metabolites [45]. Furthermore, recovering cells suppress the isocitrate (*icd*) to fumarate (*sdhA*) steps of the tricarboxylic acid (TCA) cycle while at the same time upregulating the glyoxylate bypass (Fig. 1) [45]. Importing biosynthetic precursors and differentially regulating TCA cycle activity following ionizing radiation could provide biosynthetic intermediates needed for recovery without generating high levels of ROS (oxidative stress). Although wild-type *D. radiodurans* cannot utilize most exogenously provided TCA cycle intermediates for growth [31,46], the TCA cycle genes (including genes for its glyoxylate bypass) identified by annotation [3] (Fig. 1) are functionally expressed based on our whole proteome [47] and genome expression analyses [45]. We find that four genes (*nadA-BCD*) required for nicotinamide adenine dinucleotide (NAD) biosynthesis are absent (Fig. 1) [3], and consistent with this prediction, *D. radiodurans* is dependent on exogenous nicotinic acid for growth [31].

A striking informatic (Fig. 1) and experimentally determined feature of wild-type *D. radiodurans* is its inability to utilize inorganic sulfate (Fig. 1, CysJ is missing), which results in its dependence on an exogenous source of sulfur-containing amino acids for growth [31,46]. We find that methionine alone in addition to an Embden–Meyerhof–Parnas (EMP) substrate (e.g., fructose) and nicotinic acid will support growth in defined medium (31), however, optimal growth of *D. radiodurans* in defined minimal medium additionally requires branched-chain and aromatic amino acids at >100 μg/ml (total amino acids) [46]. Other deinococci were similarly tested (Table 2) showing that the indicated strains also are dependent on methionine and NAD, and cannot utilize inorganic sulfate, suggesting that this metabolic configuration contributes to their resistance phenotypes.

We have also examined TCA cycle regulation in non-irradiated, nutrient-limited *D. radiodurans* grown in defined minimal medium [2,46] versus undefined rich medium (TGY) (Table S2 in the Appendix). For both growth conditions, 15 TCA cycle gene expression levels were examined by real time-polymerase chain reaction (RT-PCR). Notably, in defined minimal medium, only one gene (*aceA*) was statistically significantly upregulated. Six genes displayed no significant changes (*gltA*, *sucB*, *lpd*, *sucD*, *fumC*, and *aceB* DR1155); and eight genes were significantly downregulated (*acnA*, *icd*, *sucA*, *sucC*, *sdhB*, *sdhA*, *aceB* (DRA0277), and *mdh*) (Fig. 1). Thus, the response of the TCA cycle in *D. radiodurans* to nutrient-depletion or radiation is similar, with upregulation of isocitrate lyase (*aceA*) observed in both conditions (Table S2) [45]. Taken collectively, our results

Table 2
Utilization of carbon substrates by *Deinococci* grown in [a] defined minimal medium

Substrates	Species					
	D. radiodurans (ATCC BAA-816)	[b] *D. radiodurans /sodA*$^{-}$	[c] *D.radiodurans* (7b-1)	[d] *D. radiopugnans*	[e] *D. murrayi*	[f] *D. proteolyticus*
[g,h] Substrates						
Fructose + 7 amino acids	+++	+++	+++	+++	+++	+++
Fructose + Met + NAD	++	++	++	++	+/–	+/–
Fructose (–aa) + NAD	–	–	–	–	–	–
Fructose (–NAD) + Met	–	–	–	–	–	–
Pyruvate + Met + NAD	++	++	++	–	–	+/–
Acetate + Met + NAD	++	++	+++	–	+/–	+/–
α-Ketoglutarate + Met + NAD	–	–	–	–	–	–
Succinate + Met + NAD	–	+/–	+/–	–	–	–
Fumarate + Met + NAD	–	+/–	+/–	–	–	–
Malate + Met + NAD	–	+/–	+/–	–	–	–
Oxaloacetate + Met + NAD	+/–	+/–	++	–	–	+/–
Fructose + ammonium sulfate + NAD	–	–	–	–	–	–

[a] Defined minimal medium [2] was used to test the growth requirements of the indicated strains. The current table is derived from growth assays conducted as part of work reported previously [31].

[b] The superoxide dismutase A mutant (KKW7004) does not encode *sodB*, and was reported previously [58].

[c] Strain 7b-1 was isolated in 2000 from an environmental US Department of Energy radioactive waste site [30].

[d] (DSM 12027) [60].

[e] (DSM 11303) [62].

[f] (DSM 20540) [61].

[g] Growth on substrates compared to TGY, tryptone/glucose/yeast extract (rich) medium [2]: +++, good; ++, moderate; +/–, poor; –, absent.

[h] *Notes and abbreviations*: Fructose + 7 amino acids, fructose plus Met, His, Cys, Lys, Asp, Trp, and Pro, each @ 50 μg/ml; +Met (100 μg/ml), only methionine added; –aa, no amino acids added; +NAD, nicotinic acid added (1 μg/ml); –NAD, no nicotinic acid added; ammonium sulfate added to 15 mM.

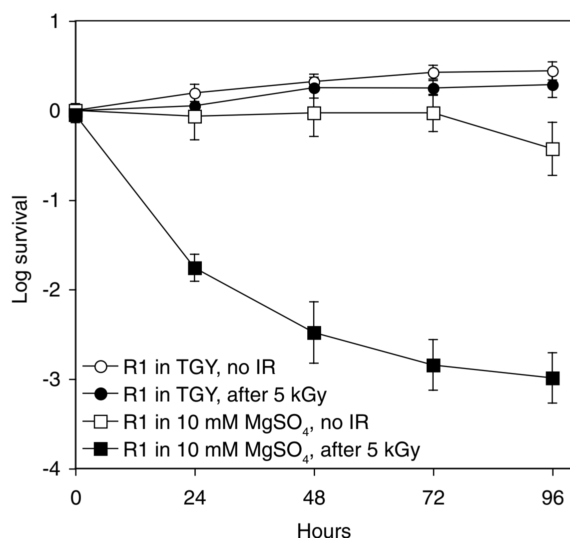

Fig. 4. Irradiated *D. radiodurans* requires nutrient conditions for recovery. Each data point is derived from three biological replicates, and standard deviations are shown. IR, ionizing radiation. See Appendix for experimental details.

indicate that *aceA* is a potential new target to control recovery from radiation injury.

Manganese(II) accumulation in *D. radiodurans* is energy-dependent [2] and the inhibitory effect of nutrient-depletion on recovery of irradiated *D. radiodurans* is documented [2,46,48,49]. However, a recent report by Harris et al. [50] claims that γ-irradiated wild-type *D. radiodurans* (R1) is able to maintain viability during prolonged incubation in the absence of nutrients in 10 mM $MgSO_4$. We were not able to reproduce those findings and it is not clear what the conditions could have been that gave rise to those results. As expected in the absence of nutrients, incubation of irradiated (5 kGy) logarithmic-phase *D. radiodurans* (ATCC 13939) in 10 mM $MgSO_4$ caused a ~1000-fold decrease in viability after 4 days (Fig. 4). Further, the exponential loss of viability of irradiated ATCC 13939 in $MgSO_4$ is essentially the same as reported for irradiated $MgSO_4$-treated mutant $ddrA^-$ (DR0423), described as encoding part of a DNA end-protection system [50]. Thus, DdrA does not appear to facilitate cell survival when energy sources are lacking; the ionizing radiation dose yielding 10% CFU (colony forming unit) survival (D_{10}) of $ddrA^-$ in rich medium is 8–9 kGy [50] compared to ~12 kGy for the wild-type.

5. Criteria for selecting organisms for comparison

Genome-based comparisons could help define how Mn homeostasis and the configuration of metabolic systems involved in energy production influence recovery, and highlight the possible nature of such processes during the evolution of environmentally robust microorganisms. There has been some confusion in defining what constitutes radiation resistance in bacteria. In assessing the resistance of bacterial cultures, failure to correct for cell-grouping (aggregation) has resulted in exaggerated reports of resistance. One approach to evaluating cells for resistance to ionizing radiation involves growth in rich medium, irradiation on ice and recovery on fresh medium, where survival frequencies are determined by dilution of irradiated cultures and colony-counts on nutrient agar plates [2]. When cells in liquid culture grow singly, death of one cell will correlate with the loss of one colony. However, where bacteria cluster in groups of cells (e.g., Fig. 1), each constituent viable cell of a cluster must be killed to eliminate a CFU. For a culture comprised of cells grouped as four or more, the shoulders of survival curves are significantly increased and the effect of cell-grouping on resistance values must be compensated [2]. Furthermore, as the cell-grouping number increases the cell-survival characteristics of an irradiated organism are expected to increase as a result of the effects imposed by oxygen limitation, where cells inside a cluster are shielded from oxygen-effects [51]. One example of an organism that might be substantially less resistant than reported is *Kineococcus radiotolerans*, which grows in liquid rich medium as very large clumps (>100 cells/CFU) [52]. The growth physiologies (cell-grouping) for the organisms listed in Table 1 have been characterized and the cell-survival frequencies have been adjusted appropriately [2]. Therefore, based on the most resistant and sensitive bacteria yet reported (Table 1), we present genome comparisons between *D. radiodurans* and *S. oneidensis* viewed from the perspective of systems that produce and defend against oxidative stress (Table 3).

6. Genome comparisons

At least four bacteria reported to be radiation resistant (*D. radiodurans*, *D. geothermalis*, *Lactobacillus plantarum* and *Rubrobacter xylanophilus*) have been subjected to genome sequencing [3] (http://www.jgi.doe.gov/), and comparative analyses support that their DNA repair systems are encoded by a variety of genes which have functional homologs in other prokaryotic species [2]. However, no shared group of uncharacterized genes has been identified in those sequenced organisms that might comprise an expanded gene core involved in recovery. Further, DNA repair systems identified in *D. radiodurans* appear less complex and diverse than those reported for *S. oneidensis* [2]. Viewed in this context, it is possible that *D. radiodurans* and other radioresistant bacteria use relatively conventional DNA repair and protection systems, but with greater efficiency than other organisms.

Table 3
Predicted systems that produce and protect against oxidative stress

[a] COG #	COG/protein annotation	*D. radiodurans*, 3.28 Mb (3182 ORFs)	*S. oneidensis*, 5.1 Mb (4472 ORFs)	Comments
Protein groups associated with the production of ROS				
	Cytochromes (all types) (see also Table 1)	10	53	Binary profiles for all types of cytochromes, flavoproteins, Fe-S domains and NADH/NADPH domains were taken from the [b] CDD database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD = search&DB = cdd). *D. radiodurans* and *S. oneidensis* were searched for these domains using profiles with default parameters as described previously [53]
	Flavoproteins	39	59	
	Iron–sulfur proteins	32	53	
	NADPH- and NADH-dependent enzymes	102	159	
Transport of protein precursors which might reduce biosynthetic demands and suppress the production of ROS				
	Peptide transporters	18	4	The approximate total number of transporters (for ABC transporters, each subunit was calculated separately)
	Aminoacid transporters	48	41	The approximate total number of transporters (for ABC transporters, each subunit was calculated separately)
	Subtilisin-like serine proteases	10	6	See text and Fig. 5 [3]
Proteins associated with the production of ROS				
COG1053	Succinate dehydrogenase/ fumarate reductase, flavoprotein subunit: fumarate reductase, FrdA	0	SO0398	FAD-containing enzymes are responsible for the catalytic interconversion of fumarate and succinate; fumarate reductase is used in anaerobic growth; in operon with SO0396 (*frdC*), SO0397 (*frdC*) and SO0399 (*frdB*)
COG1053	Succinate dehydrogenase, SdhA	DR0952	SO1928	Succinate dehydrogenase is used in aerobic growth. In operon with DR0951, DR0953 & DR0954; and SO1927 (*sdhC*) & SO1929 (*sdhB*)
COG1053	Fumarate reductase, IfcA	0	SO1421	Flavocytochrome that is induced during anaerobic respiration of Fe(III) by *Shewanella frigidimarina*; individual (not in cluster)
			SO4620	
COG1053	Fumarate reductase flavoprotein subunit precursor	0	SO0970	Individual (not in cluster)
COG1053	Flavocytochrome *c* flavin subunit	0	SO3301	In cluster with SO3300 cytC subunit
			SO1414	In operon with SO1413 4
			SO3624	In operon with SO3623 4
			SO3058	In operon with SO3056 4
COG0029	Aspartate oxidase, NadB	0	SO1341	Catalyzes the oxidation of L-aspartate to iminoaspartate; monomer; individual (not in cluster)
COG0369	Sulfite reductase, alpha subunit (flavoprotein), CysJ	0	SO3738	In operon with SO3736 (*cysI*), hemoprotein beta-component
Proteins that defend against ROS, participate in repair of damaged molecules (non-DNA) and redox regulation				
COG0605	Superoxide dismutase, SodA/B	1 (DR1279)	1 (SO2881)	Removes $O_2^{\bullet -}$
COG2032	Cu/Zn superoxide dismutase, SodC	2 (DR1546, DRA0202_1)	0	Removes $O_2^{\bullet -}$
COG0753	Catalase,KatE/A/B	3 (DRA025, DR1998, and related DRA0146)	1 (SO1070)	Removes H_2O_2
COG0376	Catalase (peroxidase I), KatG	0	2 (SO0725, SO4405)	Removes H_2O_2; activated by SoxR

COG2837	Predicted iron-dependent peroxidase	1 (DRA0145)	1 (SO0740)	Removes hydroperoxides
COG0386	Glutathione peroxidase	0	2 (SO1563, SO3349)	Removes hydroperoxides (may contain redox active selenocysteine residue in the active site); *btuE*.
COG1858	Cytochrome c peroxidase,MauG	1 (DRA0301)	1 (SO2178)	Catalyzes the peroxidative oxidation of azurin and cytochrome *c*551. Likely provides protection against toxic peroxides.
COG0450	Peroxiredoxin, AhpC	1 (DR2242)	1 (SO0958)	Peroxiredoxin family of proteins; might represent a major line of defense against hydroperoxide damage; and removes organic hydroperoxides at the expense of thiols
COG0678	Peroxiredoxin	0	1 (SO4640)	
COG1225	Peroxiredoxin	2 (DR0846, DR1209)	2 (SO1381, SO1877)	
COG2077	Peroxiredoxin	0	1 (SO3341)	
COG0225	Peptide methionine sulfoxide reductase, MsrA	1 (DR1849)	2 (SO2337, SO2588_2)	Repair of oxidized proteins (reduction of protein-bound methionine sulfoxide back to methionine via a thioredoxin-recycling process)
COG0229	Conserved domain frequently associated with peptide methionine sulfoxide reductase, MsrB	1 (DR1378)	1 (SO2588_1)	Repair of oxidized proteins (reduction of protein-bound methionine sulfoxide back to methionine via a thioredoxin-recycling process)
COG0583	Transcriptional regulator, LisR family (OxyR)	2 (DR0615, possible distant relationship)	52 (SO1328)	DR0615 has similarity to OxyR; oxidized OxyR can activate genes responding to oxidative stress
COG0789	Predicted transcriptional regulators (SoxR)	7 (DR2519)	5 (SoxR-SO1687, more distant than DR)	Phylogenetic tree shows that DR2519 is probably an ortholog of SoxR; SoxR activates *soxS* transcription
COG1304	Isopentenyl-diphosphate delta-isomerase	2 (DR1087)	0	Carotenoid biosynthesis
COG1562	Phytoene/squalene synthetase	1 (DR0862)	0	Carotenoid biosynthesis
COG1233	Phytoene dehydrogenase and related proteins	7 (DR0861)	0	Carotenoid biosynthesis
No COG	Lycopene cyclase, superfamily: FAD/NAD(P)-binding protein	1 (DR0801)	0	Carotenoid biosynthesis
COG0142	Octaprenyl-diphosphate synthase, IspB	1 (DR0932)	1 (SO3653) 1	Carotenoid biosynthesis
	Geranylgeranyl pyrophosphate synthase, IspA	1 (DR1395)	(SO1526)	
COG0584	Glycerophosphoryl diester phosphodiesterase	3	1	Esterase/lipase, class hydrolase, helps maintain integrity of cellular membranes under oxidative stress conditions
COG2267	Lysophospholipase	2	4	Esterase/lipase, class hydrolase, helps maintain integrity of cellular membranes under oxidative stress conditions
COG2272	Carboxylesterase type B	2	0	Esterase/lipase, class hydrolase, helps maintain integrity of cellular membranes under oxidative stress conditions
COG2755	Lysophospholipase L1 and related esterases	0	1	Esterase/lipase, class hydrolase, helps maintain integrity of cellular membranes under oxidative stress conditions
COG0657	Esterase/lipase	5	2	Esterase/lipase, class hydrolase; acts on ester bonds; carboxylic ester hydrolases; phospholipid degradation; glycerolipid metabolism; helps maintain integrity of cellular membranes under oxidative stress conditions
COG1166	Arginine decarboxylase	1 (DR0243)	1 (SO1870)	Polyamine biosynthesis (agmatine); polyamines inhibit the toxic effects of oxygen
COG0010	Arginase/agmatinase/formimionoglutamate hydrolase, arginase family	1 (DRA0149)	0	Polyamine biosynthesis (putrescine); polyamines inhibit the toxic effects of oxygen; putrescine is a modulator of *oxyR* transcriptional activity

(*continued on next page*)

Table 3 (*continued*)

[a] COG #	COG/protein annotation	*D. radiodurans*, 3.28 Mb (3182 ORFs)	*S. oneidensis*, 5.1 Mb (4472 ORFs)	Comments
COG1982	Arginine/lysine/ornithine decarboxylases	0	1 (SO0314)	Polyamine biosynthesis (putrescine); polyamines inhibit the toxic effects of oxygen; putrescine is a modulator of *oxyR* transcriptional activity
COG4262	Predicted spermidine synthase with an N-terminal membrane domain	0	1 (SO3763)	Polyamine biosyntheses (spermidine); polyamines inhibit the toxic effects of oxygen
COG0241	Spermidine synthase	1 (DR1052)	1(SO0960)	Polyamine biosynthesis (spermidine); polyamines inhibit the toxic effects of oxygen
Mn- and Fe-dependent systems				
COG1914	NRAMP family transporter	1 (DR1709)	0	Mn-homeostasis: transport [2]
COG1121	ABC-type Mn/Zn transport systems, ATPase component	1 (DR2284)	0	Mn-homeostasis: transport [2].
COG1108	ABC-type Mn/Zn transport systems, permease component	1 (DR2283)	1 (SO0566)	
COG0803	ABC-type Mn/Zn transport systems, perplasmic component	1(DR2523)	1 (SO0565)	
COG1321	Mn-dependent transcriptional regulator [2]	1 (DR2539)	0	Mn-homeostasis: regulation
COG3486	Lysine/ornithine N-monooxygenase	0	SO3030	Fe-homeostasis: siderophore biosynthesis; alcaligin biosynthesis
No COG	Siderophore biosynthesis protein	0	SO3031	Fe-homeostasis: siderophore biosynthesis; alcaligin biosynthesis
COG4264	Siderophore synthetase component	0	SO3032	Fe-homeostasis: siderophore biosynthesis; alcaligin biosynthesis
COG1528	Ferritin-like protein, Ftn	0	1 (SO0139)	Fe-homeostasis: iron storage
COG1629	Outer membrane receptor proteins, mostly Fe transport	0	12	Fe-homeostasis: transport
COG4771	Outer membrane receptor for ferrienterochelin and colicins	0	9	Fe-homeostasis: transport
COG0810	Periplasmic protein TonB, links inner and outer membranes	0	4	Fe-homeostasis: transport; TonB-ExbBD complex provides energy for transfer of ferrisiderophores to the periplasm via the outer membrane
COG4772	Outer membrane receptor for Fe(III)-dicitrate	0	1 (SO1102)	Fe-homeostasis: transport
COG4773	Outer membrane receptor for ferric coprogen and ferric-rhodotorulic acid	0	1 (SO3033)	Fe-homeostasis: transport
COG0783	DNA-binding ferritin-like protein (oxidative damage protectant),Dps	2 (DR2263, DRB0092)	1 (SO1158)	Fe-homeostasis: iron storage. Protects DNA from oxidative damage and induced by OxyR
COG1120	ABC-type cobalamin/Fe(III)-siderophores transport systems, ATPase components	2 (DR2590, DRB0121)	1 (SO1033)	Fe-homeostasis: transport

COG4559	ABC-type hemin transport system, ATPase component	1 (DRB0016)	1 (SO3675)	Fe-homeostasis: transport
COG0614	ABC-type Fe(III)-hydroxamate transport system, periplasmic component	5	1 (SO3709)	Fe-homeostasis: transport
COG0609	ABC-type Fe(III)-siderophore transport system, permease component	4	2 (SO1034, SO3674)	Fe-homeostasis: transport
COG2375	Siderophore-interacting protein	2 (DRB0017, DRB0124)	0	Fe-homeostasis: transport
COG1918	Fe(II) transport system protein A	1 (DR1220)	1 (SO1783)	Fe-homeostasis: Fe(II) transport system (active under anaerobic conditions)
COG0370	Fe(II) transport system protein B	1 (DR1219)	1 (SO1784)	Fe-homeostasis: Fe(II) transport system (active under anaerobic conditions)
COG0735	Fe(II)/Zn(II) uptake regulation proteins	1 (DR0865)	1 (SO1937)	Fe-homeostasis: regulation; DR0865 is similar to *zur* of *B. subtilus*

[a] COG, clusters of orthologous groups database resources of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). The number of proteins identified for each COG are indicated.

Recently, four predicted genes of *D. radiodurans* (DR0003, DR0070, DR0326 and DR0423) were identified by transcriptional profiling following ionizing radiation or desiccation as the most highly induced [14,45,50]. These genes have been disrupted, and the mutants characterized for resistance to ionizing radiation. Remarkably, the radiation resistance of the corresponding mutants (ddrC, ddrB, ddrD and ddrA, respectively) remained very high [14,45,50]. In contrast, we have shown that lowering the intracellular Mn concentration of *D. radiodurans* by a factor of just 3.3 substantially reduced the D_{10} cell-survival value [2]. Since high intracellular Mn(II) levels serve as antioxidants in several phylogenetically distinct organisms [27–29], we have proposed that Mn(II)-accumulation in *D. radiodurans* facilitates cellular repair processes after irradiation or desiccation by providing protection from metabolism-induced ROS [2]. We extend our hypothesis here to include consideration of how the metabolic configuration of *D. radiodurans* contributes to the radiation resistance phenotype.

6.1. Systems that produce oxidative stress

The prevailing hypothesis that most metabolically induced ROS are generated from within the respiratory chain has been expanded to include sources outside the respiratory chain [54]. It is known that terminal oxidases of respiring cells yield water, and not $O_2^{\bullet -}$ or H_2O_2 [55]; instead, respiratory flavins have been implicated [54,56]. One source of ROS arises from the autoxidation of respiratory dehydrogenases (Fig. 1), where adventitious transfer of electrons from reduced flavins ($FADH_2$) associated with *c*-type cytochromes to oxygen yields $O_2^{\bullet -}$ and/or $H_2O_2 + FAD^+$ [5,6]. Other sources might include flavoproteins and cytochromes not associated with the respiratory chain [54]. Therefore, the total intracellular titer of cytochromes and flavins might serve as a marker for the proclivity of cells to generate oxidative stress [56], as well as the ability of cells to survive radiation and other oxidizing conditions. Viewed in this context, the high Fe and low Mn contents of *S. oneidensis* [2] in combination with its predicted 53 cytochromes and 59 flavoproteins (Table 3) appear to make this organism ripe for Fenton-type chemistry under growth conditions where these proteins are highly expressed. In contrast, the relatively Fe-independent, Mn-rich *D. radiodurans* [2] encodes only 10 predicted cytochromes and 39 flavoproteins (Table 3); *D. radiodurans* can grow in the presence of Fe-chelators and in media with less than 0.4 μM Fe [2]. The rates at which different flavins react with oxygen vary over several orders of magnitude, depending on the extent of flavin exposure. Two enzymes with highly exposed flavins and some of the highest in vitro reactivities are fumarate reductase (FrdA) and aspartate oxidase (NadB is an Frd

homologue that lacks an Fe–S subunit) [56]. Notably, *S. oneidensis* encodes several fumarate reductases and NadB, but *D. radiodurans* does not (Fig. 1) (Table 3).

Irrespective of source, H_2O_2 is relatively inert and diffusible throughout the cell. However, when H_2O_2 encounters "free Fe(II)", Fenton-type chemistry ensues [5,6]. Therefore, conditions which liberate bound Fe(II) are extremely dangerous to metabolically active Fe-containing cells, not only because of the generation of $HO^{\bullet}$, but because the loss of Fe from iron-dependent enzymes will lead to the failure of the biochemical pathways within which they operate. Typically during irradiation, ~80% of DNA damage is caused indirectly by irradiation-induced ROS, the remaining ~20% by direct interaction between γ-photons and DNA [6]. Hydroxyl radicals are the primary product of the radiolysis of water, and in the presence of oxygen can also generate some $O_2^{\bullet-}$, and H_2O_2 by dismutation of $O_2^{\bullet-}$ [6]. In contrast, the primary ROS generated by metabolism are $O_2^{\bullet-}$ and H_2O_2 [5]. Hydroxyl radicals (standard reduction potential 2.31 V) are substantially more oxidizing than $O_2^{\bullet-}$ (−0.33 V) and H_2O_2 (−2.84 V) [6]. Since bound Fe(II), particularly from iron–sulfur (2Fe–2S, 4Fe–4S) clusters [5,56], can be released within cells when they have been oxidatively damaged [57], $HO^{\bullet}$ generated by radiolysis might increase the amount of "free Fe(II)" in cells, with the result that irradiated cells transferred to recovery medium become susceptible to sudden increases in Fe(II)-dependent oxidative stress once metabolism resumes. In contrast, cytochromes coordinate Fe extremely tightly, and likely remain functional under oxidizing conditions [5]. Notably, *S. oneidensis* encodes 65% more Fe–S proteins than *D. radiodurans* (Table 3).

6.2. Systems that defend against oxidative stress

Wild-type *E. coli* cultured under hyperbaric oxygen displays severe growth defects, which resemble those observed in SOD mutants grown in minimal medium, and are caused by $O_2^{\bullet-}$ and related ROS [5]. *E. coli* cells poisoned by $O_2^{\bullet-}$ lose their ability to assimilate sulfate, become auxotrophic for sulfurous and other amino acids, require NAD, and non-fermentable substrates (e.g., acetate, α-ketoglutarate, succinate, fumarate and malate) can no longer support growth [5]. Metabolic regulation underlying these phenotypes in *E. coli* limits TCA cycle-dependent precursor-biosynthesis, energy metabolism and the ROS that ensue [5]. Remarkably, these responses in *E. coli* lacking SOD mirror the growth deficiencies of *D. radiodurans*, which appears to have evolved a metabolic configuration that constitutively suppresses the generation of ROS (Fig. 1); wild-type *D. radiodurans* is highly dependent on methionine and other amino acids for growth, the NAD biosynthetic pathway is disrupted, sulfate assimilation is blocked, and cells are unable to utilize α-ketoglutarate, succinate, fumarate or malate for growth (Fig. 1) (Table 2). Add to this the intracellular accumulation of Mn(II) as an antioxidant [2], and it is perhaps not surprising that the role of SOD has been marginalized in *D. radiodurans*; *D. radiodurans* lacking SodA/B (KKW7004 [3,58]) has similar growth requirements (Table 2) and resistance to ionizing radiation as the wild-type [2,58]. In this context, we note that *D. radiodurans* encodes 3 catalases with some of the highest activities reported for any bacteria [42], and this would serve to remove H_2O_2 generated by non-enzymic Mn(II)-based dismutation of $O_2^{\bullet-}$. *S. oneidensis* also encodes 3 catalases but is sensitive to conditions known to produce H_2O_2 [2,44]. It is unlikely that the metabolic configuration of *D. radiodurans* (ATCC BAA-816) (Fig. 1, Table 2) is the product of mutations accumulated during maintenance under laboratory conditions since its isolation in 1956 [59]. *Deinococcus radiopugnans* (isolated 1963) [60], *Deinococcus proteolyticus* (1973) [61], *Deinococcus murrayi* (1997) [62], and another strain of *D. radiodurans* (7b-1) isolated from sediment samples collected in 2000 [32] have very similar nutrient requirements (Table 2), with the conclusion that this metabolic configuration is prevalent among *Deinococcus* species.

Table 3 contrasts the systems in *D. radiodurans* and *S. oneidensis* predicted to contribute to the defense against oxidative stress, with the most prominent differences summarized as follows: *D. radiodurans* encodes the Mn(II) transporter Nramp and a putative Mn-dependent transcriptional regulator (DR2539) [2], but *S. oneidensis* encodes neither and does not accumulate Mn(II) [2]. The absence of accumulated Mn(II) in the Fe-rich *S. oneidensis* might make this organism highly susceptible to oxidative stress, and it is noteworthy that *S. oneidensis* encodes 52 proteins predicted to be related to the LysR family, which includes OxyR, compared to just 2 in *D. radiodurans* (Table 3); OxyR is a global transcriptional regulator involved in activating genes that fight oxidative stress [6]. Carotenoids have also been implicated in protection from oxidative stress [63], and the red-pigmented *D. radiodurans* encodes 13 predicted genes involved in carotenoid biosynthesis compared to 2 in *S. oneidensis*. *D. radiodurans* also encodes 18 peptide transporters and has numerous expanded protein families for branched-chain amino acid transport, many of which are upregulated during recovery from radiation [45]. In comparison, *S. oneidensis* encodes 4 peptide transporters and displays no auxotrophies for amino acids, sulfur or NAD [1,2]. Although there is currently no evidence to support that proteases might facilitate recovery from ionizing radiation, we note that at least 10 complete open reading frames with considerable sequence similarity to secreted subtilisin-like proteases of *B. subtilis* have been identified in *D. radiodurans* [3], which might relieve its amino acid auxotrophies. *D. radiodurans* growth was tested on protease-indicator

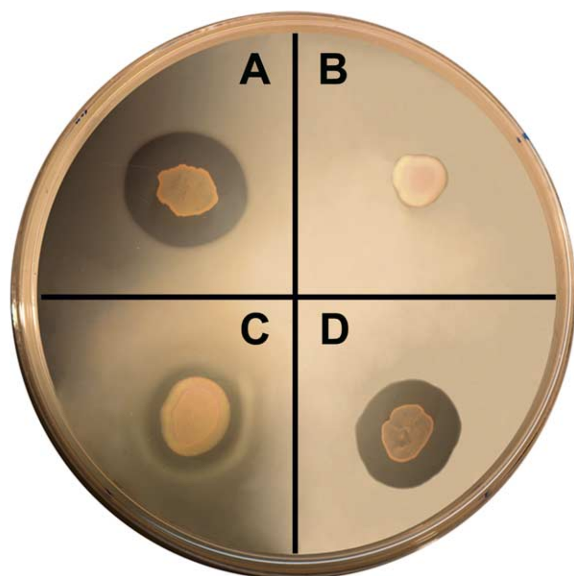

Fig. 5. Protease secretion assay on an indicator plate containing skimmed milk. (A) *D. radiodurans* (7b-1 [30]). (B) *E. coli* (strain K-12) (MG1655). (C) *S. oneidensis* (MR-1) (ATCC 700550). (D) *D. radiodurans* (ATCC BAA-816). See Appendix for experimental details.

plates containing skimmed milk, and showed luxuriant growth and generated large halo-shaped areas of clearing around colonies engaged in protein hydrolysis (Fig. 5A and D); *S. oneidensis* is predicted to encode 6 subtilisin-like proteases, and showed less protease secretion (Fig. 5C). Although, *S. oneidensis* encodes a similar assortment of catalases and peroxidases as *D. radiodurans* [2], *S. oneidensis* is exceptionally sensitive to conditions that produce H_2O_2, which include UV and ionizing radiation, and desiccation [2,44].

7. Conclusion

In the laboratory, the Fenton reaction is readily demonstrated by adding ferrous chloride to H_2O_2, which causes intense oxygen-effervescence; in comparison, manganous chloride does not. Given the structural similarities between Fe(II) and Mn(II) atoms, no great difference in reactivity with H_2O_2 is predicted, yet Mn(II) does not actively participate in Fenton-type chemistry [33]. We have reported a strong correlation between the intracellular Mn/Fe concentration ratio and the resistance to ionizing radiation and desiccation for a variety of phylogenetically distinct bacteria, and have proposed that Mn(II) ions accumulated in bacteria serve as antioxidants that reinforce enzymic systems which defend against oxidative stress during recovery [2]. For Fe-rich, Mn-poor cells such as *S. oneidensis*, death at low doses of ionizing radiation might not be caused by DNA damage inflicted during irradiation but instead by the release of Fe(II) and consequent toxic byproducts of energy-metabolism after irradiation. In addition, oxidative damage to DNA in *S. oneidensis* during recovery might induce lytic prophages, as proposed following recovery from UV radiation (200–290 nm) [44]. Conversely, *D. radiodurans* irradiated to high doses might be able to prevent bursts of oxidative stress at the onset of recovery with accumulated Mn(II), allowing repair systems and enzymic ROS-defenses to be expressed with little interference from metabolism-induced ROS. Other recent reports also attribute the release of Fe(II) and Fenton-type chemistry to radiation toxicity. Reelfs et al., (2004) [40] have reported the immediate release of labile "free Fe(II)" in skin cells as a result of oxidative damage during UV irradiation; Mn-based SOD-mimetic compounds [33,35,37,38] and Fe-chelators [40,64] have been shown to be effective in promoting radiorecovery in animals [65–67]; and it has been proposed that the radioresistance of the human osteosarcoma cell line HS-Os-1 is related to the strong scavenging of the cells for ROS following irradiation [68].

This view that the release of bound Fe(II) in irradiated cells is a primary cause of death could be tested further in sensitive versus resistant organisms by examining (i) the relationship between radiation dose and the size of the intracellular "free Fe(II)" pool, (ii) intracellular Mn/Fe localization, (iii) how genes that participate in Mn/Fe homeostasis, metal reduction, metabolism (e.g., *aceA*) and phage-induction affect resistance, (iv) the production of ROS and resistance of *S. oneidensis* under aerobic versus strictly anaerobic conditions, (v) the extent to which organisms can be made more resistant by increasing their intracellular Mn/Fe concentration ratios, and (vi) the effectiveness of Fe chelators, Mn-based catalase- and SOD-mimetic compounds, and other antioxidants (e.g., vitamins E and C) to control recovery from radiation injury.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.femsre.2004.12.007.

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