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Intracellular Copper Accumulation Enhances the Growth of *Kineococcus radiotolerans* during Chronic Irradiation

C.E. Bagwell* and C.E. Milliken

Environmental Sciences and Biotechnology, Savannah River National Laboratory, Aiken, SC, USA

*Corresponding author, Mailing address: Savannah River National Laboratory, 227 Gateway Drive, Bldg 999-W, Aiken, SC 29803, USA. Phone: (803) 819-8465. Fax: (803) 819-8432. E-mail: Christopher.bagwell@srnl.doe.gov

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Abstract

The actinobacteria *Kineococcus radiotolerans* is highly resistant to ionizing radiation, desiccation, and oxidative stress; though the underlying biochemical mechanisms are unknown. The purpose of this study was to explore a possible linkage between the uptake of transition metals and extreme resistance to ionizing radiation and oxidative stress. The effects of 6 different divalent cationic metals on growth were examined in the absence of ionizing radiation. None of the metals tested were stimulatory, though cobalt was inhibitory to growth. In contrast, copper supplementation dramatically increased cell growth during chronic irradiation. *K. radiotolerans* exhibited specific uptake and intracellular accumulation of copper compared to only a weak response to both iron and manganese supplementation. Copper accumulation sensitized cells to hydrogen peroxide. Acute irradiation induced DNA damage was similar between the copper-loaded culture as the age-synchronized no copper control culture, though low molecular weight DNA was more persistent during post-irradiation recovery in the Cu-loaded culture. Still, the estimated times for genome restoration differed by only 1 hr between treatments. While we cannot discount the possibility that copper fulfills an unexpectedly important biochemical role in a radioactive environment; *K. radiotolerans* has a high capacity for intracellular copper sequestration, and presumably efficiently coordinated oxidative stress defenses and detoxification systems, which confers cross-protection from the damaging affects ionizing radiation.

Introduction

Environmental and endogenous sources of reactive oxygen species contribute to the damage of cellular components (19,22) and a cell's ability to efficiently and effectively repair this damage is an important determinant of survival. All bacteria are equipped with defense mechanisms for coping with DNA damage and oxidative stress; however species of the genera *Deinococcus* (1)*, Arthrobacter* (18), *Rubrobacter* (17), *Kineococcus* (37), and *Chroococcidiopsis* (6), among others, are remarkable for their ability to withstand and survive tremendous cellular insults. Compared to the majority of bacteria, which generally have relatively low thresholds for stress and tolerances for cellular damage, the extreme- resistant bacteria can survive high doses of ionizing radiation, prolonged desiccation, exposure to strong oxidants and other DNA damaging agents. Three primary models have been proposed to explain the extreme resistance phenotype; 1) conventional enzymatic defenses operating at extraordinary efficiency, 2) the involvement of novel repair functions, and 3) a highly condensed, multigenomic nucleoid (5,10,31,52). While no single hypothesis explains in full the underlying genetic complexity of the extreme resistance phenotype (i.e., 49), the preferential utilization of manganese is thus far the only biochemical strategy shown to be broadly conserved among a diverse, but not comprehensive, collection of extreme-resistant bacteria (13,14,21). This finding is important because manganese, unlike iron, does not catalyze hydroxyl radical formation through Fenton / Haber-Weiss chemistry and may also mitigate protein oxidation by scavenging oxygen radicals (14). Elemental ratios of Mn:Fe have been proposed as a potentially useful indicator of a cell's susceptibility to oxidative stress (13,21). While Mn-accumulating bacteria accrue comparable levels of DNA damage as Fe-accumulating bacteria for a given dose of γ-radiation (13), Mn appears to quench secondary chemical reactions that produce reactive oxygen species; thus, promoting the effectiveness of enzymatic repair and cell survival.

Kineococcus radiotolerans was isolated within a shielded cell work area containing highly radioactive nuclear waste at the Savannah River Site in Aiken, SC, USA (37). *K. radiotolerans* is an orange pigmented, aerobic, nonsporulating actinomycete most likely belonging to the *Kineosporiaceae* family, though the exact phylogenetic placement of this genus remains unclear. While only 3 species of the genus *Kineococcus* have been described (29,37,50), each containing only a single cultivated representative, *Kineococcus*-like organisms have been detected on masonry and lime wall paintings (44), terrestrial soils (38,48), a variety of plant samples (27), marine sediments (29), McMurdo Dry Valleys of Antarctica (47), and hot deserts (20). *K. radiotolerans* is exceptionally tolerant of environmental

stresses, withstanding the damage caused by exposure to 2M hydrogen peroxide and 17 kGy of γ-radiation (C. Bagwell, Unpublished data). The physiological determinants and molecular mechanisms that minimize and repair cellular damage in *K. radiotolerans* have not been studied, and it remains unclear whether this bacterium preferentially incorporates Mn as a means of minimizing the formation of damaging oxygen radicals, and speeding recovery and survival following environmental assaults. In this study, we examined *K. radiotolerans* for preferential utilization of different divalent cationic transition metals for a possible role in anti-oxidative defense.

Materials and Methods

Culture conditions and chemicals. *Kineococcus radiotolerans* (BAA-149) was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cultures were grown on TGY medium (1.0% tryptone, 0.1% glucose, 0.5% yeast extract) at 28°C and shaken at 150 rpm for liquid cultures. Frozen stocks were prepared using the Microbank™ Bacterial Preservation System (Pro-Lab Diagnostics, ON, Canada) and were stored at -80°C. Solutions (50mM) of various divalent cationic metals were prepared in deionized water and filter sterilized. Metal salts included ferrous ammonium sulfate, manganese chloride, zinc sulfate, cupric sulfate, cobalt sulfate, and sodium molybdate (Sigma-Aldrich, St. Louis MO).

Gamma Irradiation. Liquid cultures were grown to mid exponential phase in TGY medium or TGY medium spiked with 100μM of Fe^{2+}, Mn^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, or Mo^{2+}. In a reciprocal experimental design, aliquots (25μl) of each treatment were spread plated on TGY plating medium with or without metal supplementation (100μM), respectively. Plates were exposed to 60 Gy / hr (1 Gy = 100 rad) of ionizing radiation for 4 days at a constant temperature of 30°C and colony outgrowth was counted (colony forming units, CFU). Our use of colony forming units (CFU) is strictly defined as the total number of counted colonies per plate following experimental irradiation. Acute irradiation experiments were performed by first harvesting liquid cultures (25 ml) at mid exponential phase by centrifugation (5,000 x g, 5 min, 4°C). Cell pellets were washed in ice cold 1x PBS (pH 7.3) and suspended in 10ml, 1xPBS to stall further growth and development. Culture suspensions were irradiated at a constant temperature (30°C) to achieve a total dose of 4000 Gy. Corresponding non-irradiated control cultures

were also incubated at 30°C. Post-exposure, cell concentrates were diluted into fresh TGY medium (n=3) and allowed to recover at 28°C with shaking (150 rpm) for 6 hrs.

DNA Damage Repair. DNA damage and repair was evaluated by Pulsed-Field Gel Electrophoresis (PFGE) using a modified procedure of Kieser et al. (25). Briefly, cells pellets (1ml) were collected by centrifugation and suspended in 50µl of TE-25 sucrose buffer. The cell suspensions were combined with an equal volume of molten (55°C) 4% pulsed field certified agarose and loaded into plug molds (Bio-Rad, Hercules, Calif.). Solidified plugs were incubated in TE-25 buffer and lysozyme (2 mg/ml) at 37°C for 2 hr, then transferred to NDS buffer with Proteinase K (1 mg/ml) and incubated at 37°C overnight. Plugs were washed 3 times in TE [10 mM Tris-HCl (pH 7.4), 1 mM EDTA (pH 8.0)] for 1 hr each and stored at 4°C in 0.5M EDTA prior to electrophoresis. Samples were analyzed in a 1% pulsed field certified agarose gel and 0.5x TBE running buffer using the Chef-DR III Variable Angle System (Bio-Rad) and the following electrophoresis conditions: 6 V/cm, 120° angle, initial switch time of 70 sec and a final switch time of 130 sec for 24 hr at 14°C. Gels were stained for 30 min with ethidium bromide in 0.5x TBE and documented under UV illumination using the AlphaImager™ 3400 (Alpha Innotech).

Oxidative Stress. Cultures of *K. radiotolerans* were grown to mid exponential phase in TGY medium amended with a divalent cationic metal (100µM) as described above. Cells were harvested (1.0 ml) by centrifugation, washed in an equal volume of ice cold 1x PBS (pH 7.3), and suspended in an equal volume of 4% H_2O_2. Cell suspensions were incubated in the dark for 10 min and then inoculated into fresh TGY medium (with no metals) and incubated at 28°C with shaking (150 rpm) for 48 hrs. Recovery and growth was evaluated by protein quantification using the *DC* Protein Assay Kit (Bio-Rad, Hercules, Calif.).

Analytical methods. Total intracellular metal contents were quantified by inductively coupled plasma-mass spectroscopy (ICP-MS). Briefly, cells were harvested by centrifugation (10,000 x g, 5min, 15°C) and sequentially washed 3x each in 50mM EDTA / 1x PBS (pH 7.5; 1x PBS = 137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4) and 25mM EDTA / 1x PBS in order to remove weakly cell surface adsorbed metal. Cells were then washed 3x in PBS and immediately frozen in liquid nitrogen

and stored at -80°C. Effectiveness of the metal chelation wash steps was confirmed by spectrophotometry (7). For intracellular metal analysis, cell pellets were digested at room temperature in 0.1 ml ACS grade concentrated H_2O_2 and 0.2 ml concentrated optima grade HNO_3. The samples were then diluted with 18.2 MOhm*cm DI water and analyzed in standard mode on a Perkin Elmer-Sciex Elan DRC Plus ICP-MS according to EPA method 6020a. External calibration was performed using NIST traceable standards diluted in the same matrix as the samples and the calibration was verified against a standard with a different lot number.

Results

Effect of metals on growth during chronic irradiation. Growth experiments were conducted in order to evaluate the effect of metal supplementation. Absolute normalization of cell titers between treatments is impractical because of the extensive clumping of *K. radiotolerans* cultures growing in TGY medium; however, protein determinations and CFUs have proven to be reliably consistent methods of monitoring growth (data not shown). Protein levels and the number of colony forming units (CFUs) were determined for metal treated (100μM final concentration) cultures at 4 distinct phases of growth; early, mid, and late exponential, and stationary (data not shown). Lag times and growth yields from Fe^{2+}, Cu^{2+}, Mn^{2+}, Mo^{2+}, and Zn^{2+} treated cultures were the same as the no-metal TGY control. Co^{2+} amendments resulted in an exaggerated lag period but protein yields and CFUs at stationary phase were the same as the other metal treatments and the no-metal control. These data were used to establish that biomass yields were not drastically different between metal treatments, with the exception of Co^{2+}, in the absence of chronic irradiation.

Irradiated no-metal control cultures consistently yielded 75 CFU. This value established the baseline for which to evaluate the effects of the metal additions on growth of *K. radiotolerans* during chronic irradiation. The results in Figure 1 were interpreted conservatively by permitting a variance of ± 25 CFU around this reference line.

The effect of metal supplementation on growth during chronic irradiation (Black bars) was mostly neutral; though surprisingly, Mn^{2+} appeared to have a detrimental impact on growth. As expected, cultures plated on Co^{2+} supplemented medium yielded fewer CFUs (~1/3) than the controls, though the combination of Co^{2+} and chronic irradiation was not lethal. Conversely, pre-growth on metal amended

medium prior to irradiation (white bars) yielded different results. In this case, only Fe^{2+} supplementation was inhibitory to growth. Cobalt appeared to be more toxic to growth than chronic irradiation as growth yields improved when metal stress was relieved. Pre-growth with Mo^{2+} resulted in higher CFU yields during chronic irradiation, while the effect of both treatments combined was neutral.

The most striking results were obtained for the Cu^{2+} treatments. Not only did the Cu^{2+} primed cultures form a lawn during chronic irradiation, but the TGY grown culture also achieved a lawn of growth during chronic irradiation when Cu^{2+} was added to the plating medium. Bars for the Cu^{2+} treatments were conservatively plotted to 200 CFUs since a closer approximation of the actual CFUs could not be determined.

Growth recovery from oxidative stress. Experiments were conducted to determine whether *K. radiotolerans* cultures pre-grown on TGY in the presence of various divalent cationic metals exhibited differential sensitivity to hydrogen peroxide (Figure 2). Five percent hydrogen peroxide is lethal to exponentially grown *K. radiotolerans* cultures in TGY (data not shown), and TGY-grown control cultures resumed cell growth approximately 24h after exposure to 4% H_2O_2. Growth recovery of Fe^{2+}, Mn^{2+}, and Mo^{2+} pre-grown cultures were similar to the TGY controls, but the Mn^{2+} and Mo^{2+} treated cultures required approximately 5-10 hr longer to enter into exponential growth. No difference in protein abundance was measured for the Cu^{2+}, Zn^{2+}, and Co^{2+} grown cultures for up to 48 hr following exposure to H_2O_2.

Intracellular accumulation of metals. *K. radiotolerans* cultures were grown to early stationary phase in TGY medium amended with 0.1mM Mn^{2+}, Fe^{2+}, or Cu^{2+} and intracellular metal contents were quantified by ICP-MS (Figure 3). Biomass yields were not statistically different among each of the metal treatments ($p < 0.05$, data not shown). The TGY growth medium contained 0.2 μM both for Mn and Cu, and 5.8 μM for Fe. *K. radiotolerans* no-metal control cultures had higher levels of intracellular Fe (~50 ng / mg protein) than Mn or Cu (< 5 ng / mg protein) which capitulated the relative levels in the TGY medium. Metal supplementation of the growth medium significantly increased intracellular quantities for each of the metals examined relative to the controls ($p < 0.05$). Iron amendment resulted in only a slight increase in intracellular Fe (1.4x), while Mn^{2+} amendments increased intracellular levels by nearly 7x. *K. radiotolerans* cultures grown in TGY medium with no metal had intracellular Fe contents that

were approximately 8x higher than Cu, but cultures actively accumulated Cu when the growth medium was supplemented. Copper amendments increased intracellular Cu by 80x over the controls. It is also interesting to note that Mn^{2+} and Cu^{2+} supplementation and enhanced intracellular accumulation of these metals reduced the intracellular quantity of co-accumulated Fe.

Intracellular metal contents and ratios of transition metals in *K. radiotolerans* were compared to other radiation resistant and radiation sensitive bacteria (Table 1, see also 13). Grown in TGY medium, *K. radiotolerans* had lower intracellular Fe levels (0.86 nmol/mg protein) than any of the other radiation resistant bacteria (> 1.4). Only when grown in Fe^{2+} supplemented TGY medium (1.25 nmol/mg protein) did intracellular iron levels approximate those for *Deinococcus* spp (1.45-1.7 nmol/mg protein). Likewise, levels of Mn were also markedly lower (0.075 nmol/mg protein) than levels previously reported for other radiation resistant bacteria (>0.3 nmol/mg protein), though not as low as the radiation sensitive strains (<0.019 nmol/mg protein), but when grown in Mn^{2+}-supplemented medium levels (0.51 nmol/mg protein) exceeded those for *D. radiotolerans* (0.36 nmol/mg protein).

In general, Mn/Fe ratios for *K. radiotolerans* were more closely aligned with values reported for the radiation resistant bacteria than the very low ratios for the radiation sensitive strains, though metal levels varied considerably with growth conditions. Mn/Cu ratios were generally quite high (i.e., favorable ratio for resistance) except for cultures grown in Cu^{2+} supplemented medium, and then this ratio was more closely aligned with the Mn/Fe ratios for the radiation sensitive strains. Given the responsiveness of uptake and accumulation mechanisms for Cu^{2+}, Mn/Fe+Cu ratios were also determined. In general, ratios were more closely aligned with Mn/Fe ratios of the radiation tolerant than radiation sensitive strains, except when grown in Mn^{2+} supplemented medium, where the ratio was exceptionally high (i.e., purportedly favorable for resistance) and in Cu^{2+} supplemented medium the ratio was lower than the Mn/Fe ratio for *E. coli* (i.e., taken to indicate sensitivity).

DNA damage repair following acute irradiation. Experiments were conducted to evaluate the effects of copper accumulation on DNA elicited by acute exposure of *K. radiotolerans* cultures to ionizing radiation. PFGE was used to qualitatively evaluate the extent of radiation-induced DNA damage and repair between copper and no-metal control treatments. *K. radiotolerans* possesses 2 plasmids (183 and 14 kbp), but only the smaller plasmid is reliably resolved on these gels and thus provides a useful visual

marker to approximate the completion of genome restitution. Based on protein determinations, cell titers were equivalent between the Cu^{2+}- loaded and control cultures (Figure 4BC). In Figure 4A, the occurrence of DNA double strand breaks was γ-radiation dose dependent. Relatively low radiation doses (40 Gy) produced a full range of damaged DNA fragments in *K. radiotolerans* as indicated by a long smear, and DNA from cells exposed to 4,000 Gy was predominately visualized as low molecular weight DNA which co-migrated near the bottom of the gel. In the no-metal TGY grown control culture, the low molecular weight damaged DNA disappeared and the 14 kbp plasmid appeared within 3 hr following acute irradiation (Figure 4B). Comparable levels of radiation induced DNA damage (i.e., relative intensity of the smear) were noted for the copper loaded culture but the smear itself may have been slightly longer (Figure 4C). DNA damage repair in the acutely irradiated Cu^{2+}-loaded culture required 4 - 5 hr for the low molecular weight damaged DNA to fully disappear and for the 14 kbp plasmid to be clearly visible, though the intensity of the plasmid was notably less than in the control.

Discussion

Copper is an essential cofactor for a variety of enzymes involved in aerobic respiration and energy production; however, excess copper is toxic and thus, intracellular levels are tightly regulated by the cell (39). Copper toxicity manifests itself through indiscriminant binding to cellular ligands or competitive displacement of other metal cofactors (4,11), as well in the production of intracellular reactive oxygen species (ROS), namely hydroxyl radical, via Fenton chemistry (22,26). While copper catalyzed reduction of H_2O_2 to hydroxyl radicals can be demonstrated *in vitro* (3,16), the significance of this reaction in mediating DNA damage *in vivo* remains a controversial issue. This reaction is considered unfavorable on account of the low physiological concentrations of oxygen radicals, the virtual absence of 'free' copper inside the cell, and the maintenance of a neutral pH cytosolic environment (39, 45); however, numerous studies have demonstrated that these reactions do occur (32) and are a significant threat to cell viability and survival (26). Copper homeostasis is critical as evident by the cell's capacity for copper chelation and complex detoxification systems (15,28,36,39) which are often interconnected through transcriptional regulation of oxidative stress pathways (24,46). In Gram negative bacteria, periplasmic sequestration of metals is an important resistance mechanism to prevent accumulation of toxic levels of Cu^{2+} in the cytoplasm (8,9,32,41), but *K. radiotolerans* is Gram positive and unequivocally accumulates copper intracellularly. Moreover, heightened sensitivity to a strong oxidant

and the persistence of low molecular weight DNA following acute irradiation of copper-loaded cells is suggestive of copper-catalyzed hydroxyl radical formation.

It is unclear why *K. radiotolerans* accumulates copper but it appears that this organism possess uptake and transport mechanisms that are Cu^{2+}-specific. It would seem unlikely that *K. radiotolerans* possesses a unique, copper-dependent defense system for oxidative and radiation stress, yet more probable that cells are sufficiently equipped to sequester excess copper (or redox active metals) at high efficiency, thus preventing metal toxicity and production of reactive oxygen species. This conclusion would appear to be supported by the observation that Fe loaded cells were not differentially affected by chronic irradiation or H_2O_2 relative to the no-metal control. Possible mechanisms for copper sequestration based on preliminary genome sequence examination include the Fe-containing superoxide dismutase (SOD), glutathione (GSH), and DNA binding protein (Dps). Orthologs of putative low molecular weight Cu-induced metallothioneins or metallochaperons have not been identified in *K. radiotolerans*. Additionally, conventional copper homeostasis pathways (i.e., *cop* operon; 45) appear to be absent, though numerous heavy metal transporters and multiple copies of the *CopC* copper resistance gene were identified. Copper toxicity can manifest itself through the displacement of iron, or other metals, for specific ligands and cofactor binding sites; though, growth characteristics were unaffected by high cytoplasmic copper levels. Adaptation to copper supplemented growth medium may induce the expression of additional or redundant defense and detoxification systems that counter metal toxicity, as well as excessive production of reactive oxygen species. Intracellular sequestration may be important for copper resistance but the cupro-chaperons or enzymes involved and the potential coordination with other defense systems has not been experimentally determined.

We have repeated the experiments performed by Daly et al. (13) to evaluate whether certain transition metals might fulfill an important physiological role in the radioresistance phenotype of *K. radiotolerans* as they appear to in other extreme resistant bacteria. High levels of intracellular Mn relative to Fe have been shown to contribute to γ-radiation resistance by mitigating protein oxidation that occurs during and after irradiation (14). Conversely, the high intracellular Fe, relative to Mn, apparently contributes to the sensitivity of bacteria like *E. coli* and *S. oneidensis* to radiation and oxidative stress through the production of reactive oxygen radicals that exacerbates cellular damage. The apparent biochemical preference and utilization of a non-Fenton redox metal by the *Deinococcus* spp. and other extreme

radiation resistant bacteria tested by Daly et al. (13), though, does not satisfactorily explain the radiation tolerance of *K. radiotolerans* because Cu^{2+} does participate in Fenton / Haber-Weiss chemistry for the formation of reactive oxygen species (30). These results strongly indicate an important role for Cu^{2+} for enhancing the metabolic efficiency and / or anti-oxidative capacity in *K. radiotolerans* to compensate for the chemical reactivity of this element. It is relatively simple to envision that the copper-induced response could afford cross-protection from other stressors (particularly oxidative stress), but it is not clear how a stress response would dramatically increase energy production and growth but only when the compounded stress of ionizing radiation is applied.

Mattimore and Battista (33) postulated that mechanisms of extreme radioresistance in *Deinococcus radiodurans* did not evolve under direct selection by ionizing radiation, but more likely evolved as a consequence of selection for desiccation resistance. This explanation continues to gain credence as more examples arise (e.g., 2,20,40,47). These observations imply certain overlap among the underlying resistance mechanisms, but there may also be some important stressor-specific distinctions. Here, high intracellular levels of copper inhibited growth of H_2O_2 exposed cells, though the expectedly toxic combination of copper and γ-radiation proved stimulatory to the growth of *K. radiotolerans*. Exposure of a Cu-loaded culture to 4% hydrogen peroxide exceeded the cells capacity to effectively quench reactive oxidants, resulting in significant cellular damage, consistent with the expectation that copper catalyzed the production of oxygen radicals. The sensitivity of Cu-loaded cultures to more closely approximated physiological concentrations of hydrogen peroxide was not determined; though, we have measured heightened sensitivity of Cu-loaded cultures to methyl viologen (0.2 mM), a known producer of superoxide anion (C.E. Bagwell, Unpublished data). Thus, the levels of oxidative stress resulting from chronic irradiation of Cu-loaded cells should be less than that imposed by the H_2O_2 used in these experiments. We presume that exposure of Cu-loaded *K. radiotolerans* cultures to a strong oxidant is capable of liberating 'bound' intracellular Cu which is then available to react with lethal consequences to the cell. Consequently, Cu-dependent growth stimulation during chronic irradiation may mean that radiation-induced oxidative stress is below threshold and any oxidant produced is readily quenched, so then a beneficial role for copper is conceivable.

The relative amount of direct DNA damage resulting from acute irradiation was comparable between the Cu-loaded and control cultures, whereas Cu-dependent damage was more pronounced during post-

irradiation recovery and repair. The Cu-loaded culture may suffer from a higher level of indirect cellular damage due to Cu-dependent production of reactive oxygen species, which would interrupt the efficiency of DNA stabilization and repair. Decreased intensity of the restored plasmid in the Cu-loaded culture implies that some of the low MW DNA could not be salvaged for genome reassembly and was either degraded or exported. We presume that the type or extent of DNA damage accrued in the Cu-loaded culture was more severe than the control; however, it is interesting to note that DNA stabilization and repair functions were preserved and operated at nearly the same efficiency as the control. Daly et al. (14) have emphasized the importance of protein oxidation for bacterial radioresistance, and we hypothesize that Cu-adaptation may preferentially or differentially protect cellular proteins from oxidative damage.

Microbial communities do inhabit radioactive environments (18,23,37,42,51), and bacteria isolated from such habitats are generally much more tolerant of exposure to ionizing radiation and oxidative stress than their counterparts from environments experiencing background levels of radiation (35). Melanin producing microfungi obtained from the Chernobyl Atomic Energy Station display directional growth of hyphae towards ionizing radiation (51). Dadachova et al. (12) recently demonstrated that melanin enhanced the growth and metabolic activity of certain fungi during chronic exposure to low levels of ionizing radiation relative to non-melanized cells. Though the exact mechanism(s) is unknown; melanin may serve to shield these fungi, perhaps scavenging reactive oxygen species (43), but a role for the electron transfer properties of melanin cannot be ignored (12,34). To the best of our knowledge this study marks the first documented case whereby bacterial growth is legitimately enhanced during chronic irradiation. Here, growth conditions that were expected to prompt copper catalyzed production of oxygen radicals actually promoted the growth of *K. radiotolerans*, and this response could not be duplicated by chronic irradiation or copper supplementation alone.

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Table 1. Intracellular metal contents of extreme resistant and sensitive bacteria.

Strain	Radiation Resistance	Mn/Fe	Mn/Cu	Mn/Fe+Cu
K. radiotolerans	RT			
TGY		0.087	0.75	0.078
TGY + Fe^{2+}		0.0048	0.545	0.044
TGY + Mn^{2+}		0.91	3.778	0.734
TGY + Cu^{2+}		0.087	0.0071	0.0066
Deinococcus radiodurans ‡	RT	0.24		
Enterococcus faecium ‡	RT	0.17		
Pseudomonas putida ‡	RS	<0.0001		
E. coli ‡	RS	0.0072		
Shewanella oneidensis ‡	RS	0.0005		

K. radiotolerans cultures were grown to stationary phase and cell pellets were washed sequentially in EDTA and PBS prior to metals analysis by ICP-MS. All values are presented as metal / protein (nmol / mg). ‡ Comparative data for other bacterial strains were taken directly from Daly et al. (2004). Levels of radiation resistance were categorized by strain as either radiation tolerant, RT, or radiation sensitive, RS.

Figures

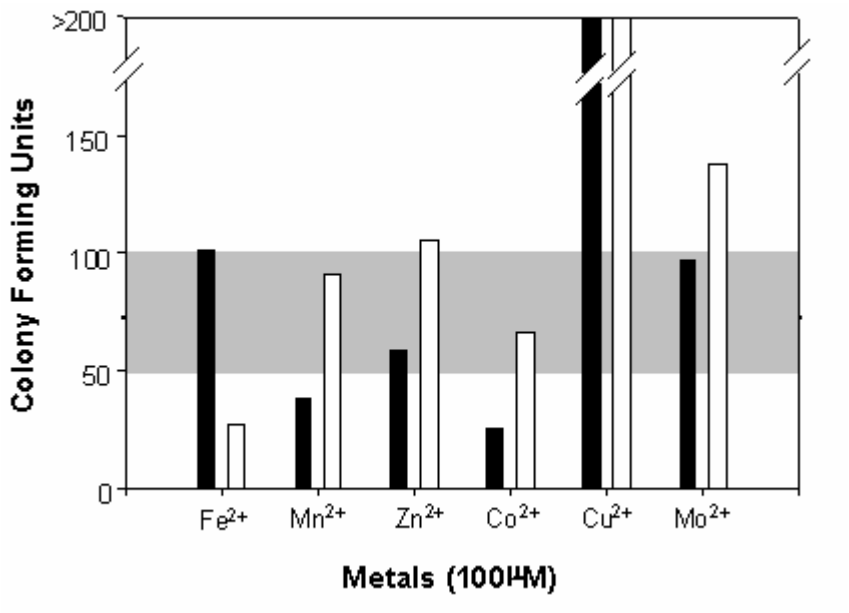


Figure 1. The effect of different transition metals on the growth of *K. radiotolerans* during chronic irradiation. Black bars indicate cultures that were grown to exponential phase in TGY medium and then streak plated onto metal supplemented TGY plates. White bars indicate cultures that were pre-grown to exponential phase in metal supplemented liquid medium and then streak plated onto no-metal containing TGY plates. The horizontal baseline (gray bar) of 75 ±25 CFUs was established from the no-metal, irradiated control cultures. All plates were irradiated for 4 days at 60 Gy/hr at a constant temperature of 30°C.

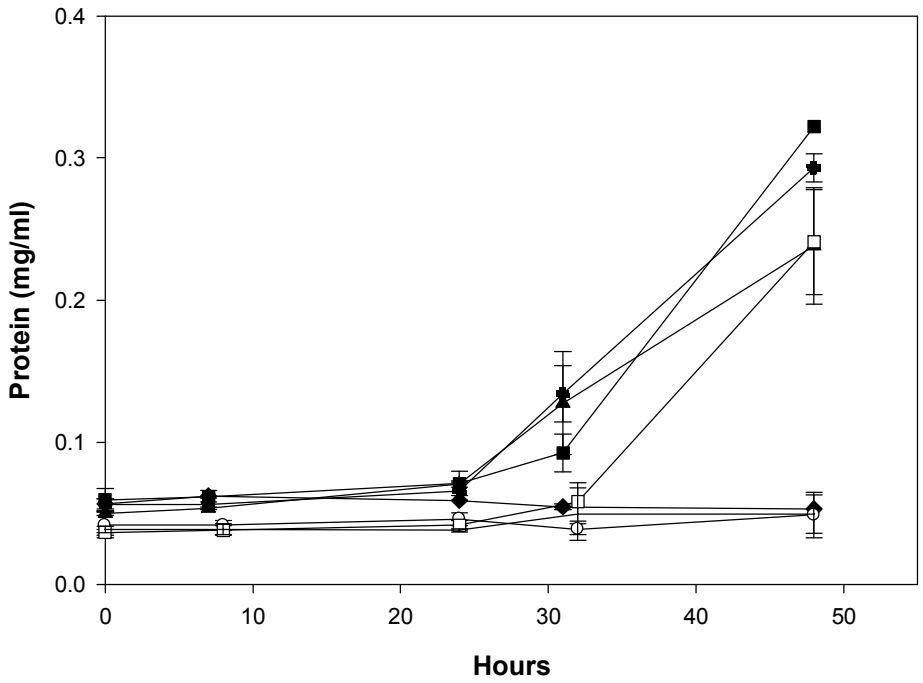
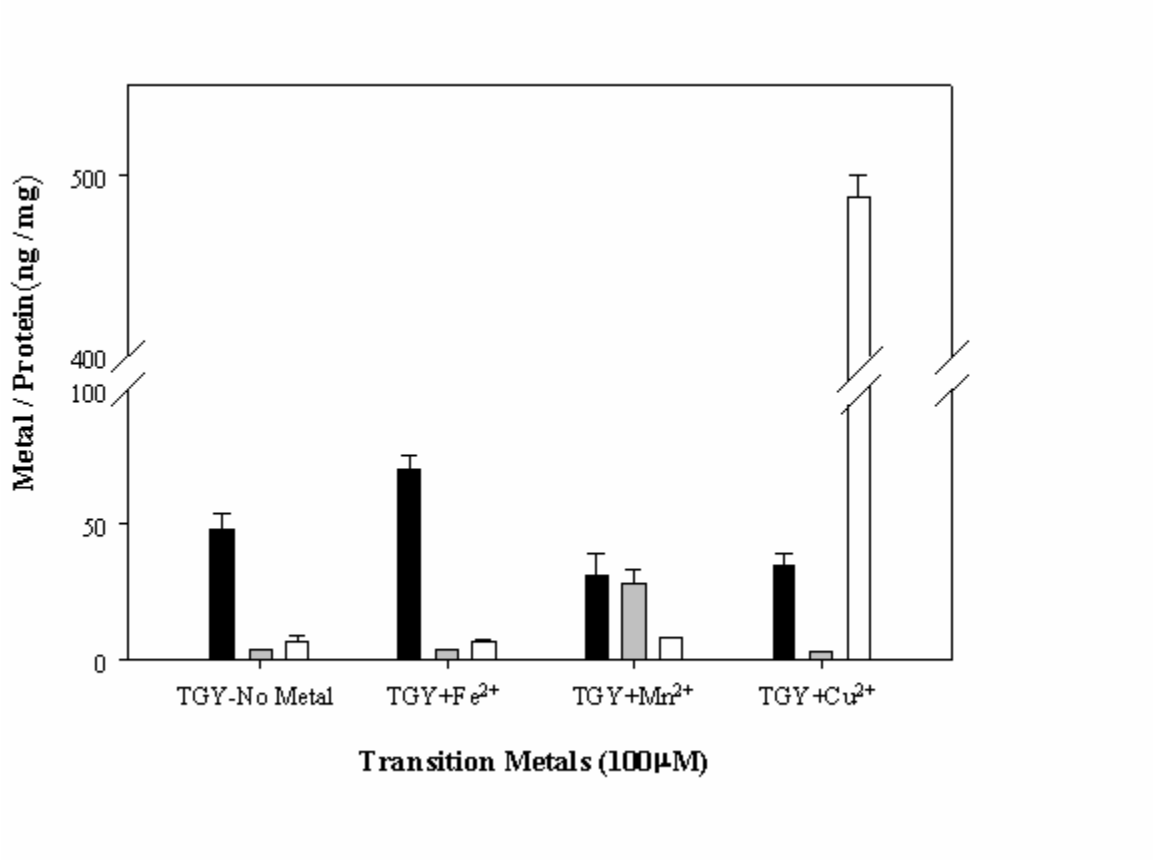


Figure 2. Recovery response of *K. radiotolerans* following H_2O_2 exposure. Cultures (n = 3) were grown to exponential phase in TGY or TGY amended with a divalent cationic metal (100μM), incubated in H_2O_2 (4%) for 10 min, and then allowed to recover in fresh TGY medium at 28°C and 150 rpm. ▲TGY (Control), ■ TGY + Mn^{2+}, ♦ TGY + Cu^{2+}, ● TGY + Fe^{2+}, o TGY + Zn^{2+}, □ TGY + Mo^{2+}, and Δ TGY + Co^{2+}.

2



4 **Figure 3.** Intracellular accumulation of transition metals in early stationary phase *K. radiotolerans* cultures. Bars indicate normalized metal content of Fe^{2+} (solid), Mn^{2+} (grey), and Cu^{2+} (white)
6 respectively in no-metal control and metal supplemented cultures (X-axis).

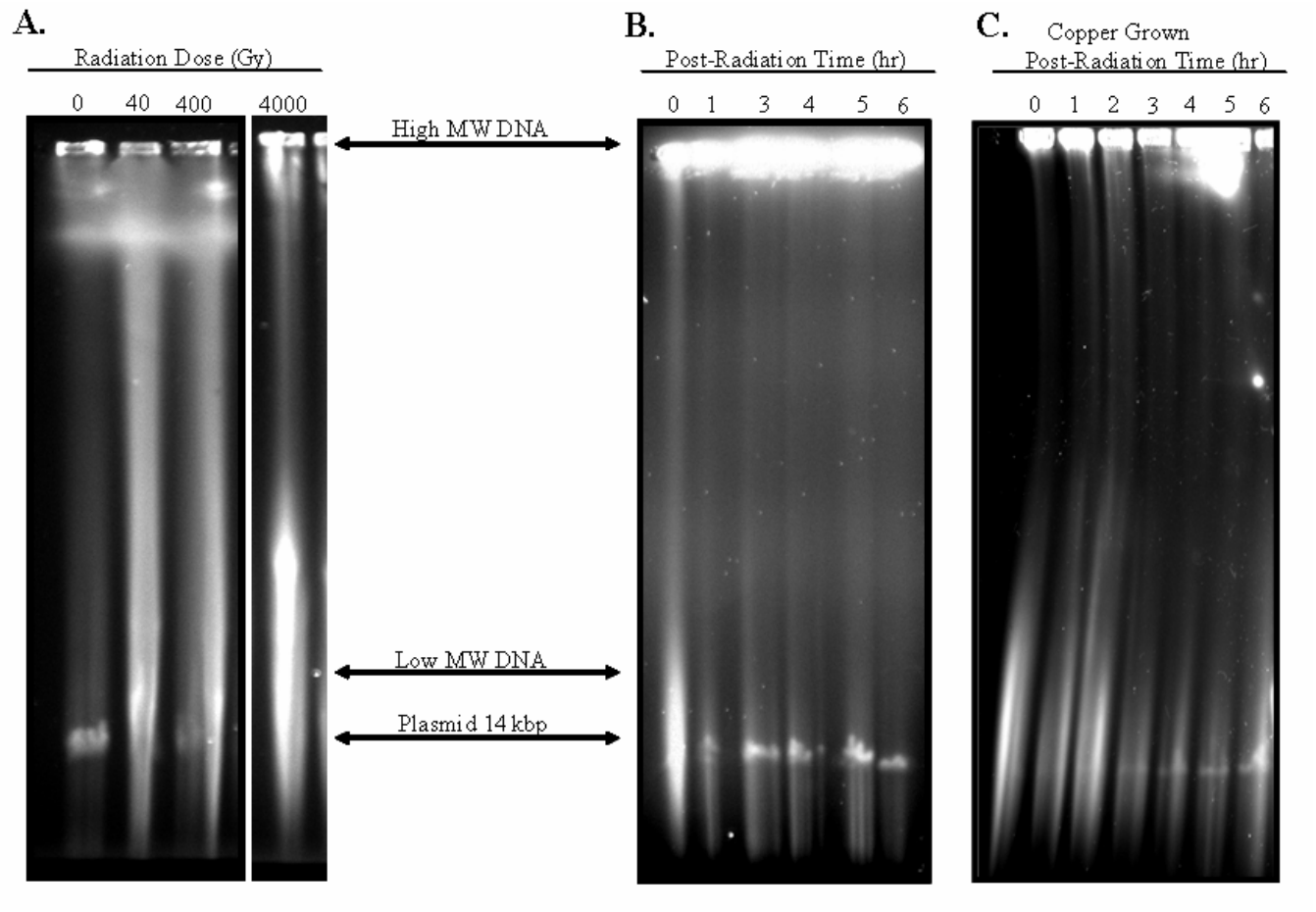


Figure 4. DNA damage repair in *K. radiotolerans* cultures following acute irradiation. Panel A, DNA damage as a function of radiation dose. Panels B and C, The timing of radiation induced DNA damage repair for control and Cu-grown cultures.