ABSTRACT

Title of Document: RESISTANCE TO IONIZING RADIATION AND OXIDATIVE STRESS IN *HALOBACTERIUM SALINARUM* NRC-1

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Oxidative stress results from environmental challenges that cause unchecked production of reactive oxygen species (ROS). We analyzed the cellular damage and stress response of the extremophile *Halobacterium salinarum NRC-1* exposed to chemical oxidants and to ionizing radiation (IR). In contrast to IR, cellular damage from $H_2O_2$ and superoxide suggested that cell death resulted from interference with major metabolic pathways rather than generalized oxidative lesions. We found that essential ROS scavenging enzymes were not necessary for *H. salinarum* NRC-1 survival to IR. Protection assays using enzyme-free cellular extracts from *H. salinarum* NRC-1 demonstrated high level of protection for protein activity but not for DNA integrity against IR. Biochemical analysis of the extracts underlined an essential role in ROS scavenging for specific nucleosides and $MnPO_4$ complexes. These studies contributed novel findings on the critical role played by non-enzymatic systems in IR resistance in *H. salinarum* NRC-1.

# RESISTANCE TO IONIZING RADIATION AND OXIDATIVE STRESS IN *HALOBACTERIUM SALINARUM* NRC-1

By

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# Dedication

This work is dedicated to Ken, Diane, and Cutler Robinson for all their love and support.

# Acknowledgements

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# List of Abbreviations

8-OH-Gua – 8-hydroxyguanine
BER – base excision repair
BSS – basal salt solution
DNPH – 2,4-dinitrophenylhydrazine
FapyAde – 4,6-diamino-5-formamidopyrimidine
FapyGua – 2,6-diamino-4-hydroxy-5-formamidopyrimidine
GC/MS – gas chromatography coupled to mass spectrometry
$H_2O_2$ – hydrogen peroxide
ICP-MS – inductively coupled plasma mass spectrometry
IR – ionizing radiation
LC-MS – liquid chromatography coupled to mass spectrometry
NER – nucleotide excision repair
PFGE – pulsed field gel electrophoresis
PQ – paraquat
ROS – reactive oxygen species
UF – ultrafiltrate

# Chapter 1: Introduction

Hypersaline environments undergo harsh periods of desiccation and rehydration that pose significant challenges to organisms that inhabit them. As water evaporates, organisms are surrounded by increasingly saline solutions until becoming encased in salt crystals, then going back into solution once water levels increase. Halophilic organisms have developed many specific adaptations to deal with this extreme environment. To maintain osmotic balance, cells can accumulate compatible solutes or high intracellular salts. The later strategy requires that organisms also have proteins specifically adapted to the high intracellular salt environment [1]. Halophiles also have efficient defense mechanisms to control damage from desiccation or UV light. The halophilic archaeon *Halobacterium salinarum* NRC-1 is adapted to some of the highest salt environments, growing optimally at 4M NaCl [1]. It has previously been demonstrated that *H. salinarum* NRC-1 is highly resistant to UV radiation as an adaptation to high solar irradiance in its environment [2]. Additionally, *H. salinarum* NRC-1 was found to be highly resistant to desiccation and ionizing radiation (IR) [3]. Desiccation resistance is likely due to the requirements of its hypersaline environment, and not resistance to IR, since no naturally occurring environments has such high radiation levels [3]. This then suggests a link between IR and desiccation resistance [3].

This link between desiccation and IR resistance has also been demonstrated in other organisms [4-6]. It has been shown that both conditions introduce DNA double

strand breaks and produce oxidative damage to proteins [5-7]. IR has long been known to produce oxidative damage, mainly through the radiolysis of water and subsequent production of hydroxyl radicals [8]. Since both resistance to IR and desiccation are often observed in the same organism, it has been inferred that oxidative damage is the main challenge posed by IR and desiccation [5].

This link between the effects of oxidative damage and the challenges posed by IR has not been clearly elucidated. As previously stated, oxidative stress to proteins have been measured in response to IR [7, 9]. DNA damage has been assessed through the amount of double strand breaks following IR and desiccation [5, 10], as well as levels of nucleoside lesions following IR [9]. This study aims to more fully explore the damage that is introduced to cellular molecules of DNA and proteins by exposure to chemical oxidants and IR using *H. salinarum* NRC-1 as a model system. It also seeks to determine the defense systems *H. salinarum* NRC-1 has in place to deal with oxidative stress and IR. This includes both enzymatic scavengers of reactive oxygen species and any chemicals capable of protecting cellular components from oxidative damage.

*Sources of oxidative stress and cellular strategies to minimize its effects*

Aerobic organisms are surrounded by oxygen and depend upon it for cellular respiration, the oxidation of organic molecules to release energy, and ultimately, their survival. It is somewhat surprising, then, that oxygen can be converted into such toxic molecules as superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide ($H_2O_2$), and

hydroxyl radicals ($^{\bullet}OH$). This can happen routinely through normal cellular respiration, which is the major source for superoxide in *Escherichia coli*, or during other cellular processes, producing hydrogen peroxide and other reactive oxygen species (ROS) [11]. ROS exposure can also result from exogenous sources, either through the release of oxidants from chemical reactions in the environment, or from production of ROS by other organisms as a defense strategy [11]. The ROS produced by endogenous and exogenous processes are capable of causing damage to cellular lipids, proteins, and DNA. Hydrogen peroxide, although less reactive than superoxide or hydroxyl radicals, is lipid soluble and readily crosses membranes. It is able to be converted to the more reactive hydroxyl radical species via Fenton's reaction [12].

Fenton's reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$$

This hydroxyl radical is reactive at diffusion rate limits and is likely the primary cause of damage to macromolecules in the cells [12]. If there are suitable reducing species available (including superoxide), $Fe^{3+}$ can be converted back to $Fe^{2+}$, resulting in a cyclical process that can produce large amounts of hydroxyl radicals in a targeted area [13]. In addition to being produced through normal respiration, superoxide can be artificially introduced to cells using chemicals such as the herbicide paraquat (N,N'-dimethyl-4,4'-bipyridinium dichloride). In the presence of $O_2$, paraquat

undergoes oxidation-reduction cycling inside the cell that results in the production of superoxide [14].

The damage introduced by superoxide and hydrogen peroxide to DNA and proteins appears to be closely dependent upon the presence of iron. Damage to DNA tends to occur due to the close proximity of $Fe^{2+}$ bound to DNA. Hydrogen peroxide undergoes Fenton's reaction in the presence of Fe and produces hydroxyl radicals. These radicals cause heavy damage to the surrounding DNA [11, 12]. This damage can often be site-related, due to iron's preferential binding to certain sequences of DNA and the position of the iron available to interact with the hydrogen peroxide [15]. Superoxide, however, causes damage to DNA indirectly through the liberation of $Fe^{2+}$ from iron-sulfur clusters of proteins [11, 16]. This free iron can bind to DNA and act as the site for Fenton's reaction to occur. It is also possible for this free iron to bind the surfaces of lipids and proteins, thus causing damage to those molecules as well [11].

Protein damage from ROS occurs mainly in proteins with exposed iron-sulfur clusters $[4Fe\text{-}4S]^{2+}$. Hydrogen peroxide and superoxide are able to bind one of the Fe atoms in these clusters and oxidize it to an unstable redox state [11]. The cluster becomes oxidized, univalently by superoxide and divalently by hydrogen peroxide, to $[3Fe\text{-}4S]^{+}$ and $Fe^{2+}$ is released and now free to participate in Fenton's reactions [11, 17]. It is also believed that hydrogen peroxide is involved in metal-catalyzed oxidation reactions that are responsible for adding carbonyl groups to proteins [12]. Metal-

catalyzed protein oxidation is often considered a "caged" process where amino acids near metal binding sites are targeted [18]. Damage is most often the result of hydrogen peroxide interacting with transition metals ($Fe^{2+}$, $Cu^{+}$, etc.) to produce hydroxyl radicals, which then react with surrounding molecules [19]. Usually transition metals are bound to protein active sites that are recessed from the surface of proteins, so these ROS-producing reactions can be sequestered from ROS scavengers in the cells, allowing the damage to propagate to amino acid residues without intervention from ROS scavengers [18]. Individual proteins seem to show different susceptibilities to this, potentially due to their abilities to bind metals necessary for the oxidation [12].

Due to the extensive effects that oxidative stress can cause to cellular components by endogenous or exogenous sources, organisms have developed a variety of coping mechanisms, including both damage avoidance and damage repair. Stress responses to oxidative damage have been discovered in many organisms, including eukaryotes such as yeast [20] and *Arabidopsis thaliana* [21], and bacteria including *Escherichia coli* [22] and *Salmonella enterica* [23]. Among the best studied systems are the two major transcriptional regulators involved in stress response to oxidative damage in *E. coli*: OxyR, which responds to hydrogen peroxide exposure, and SoxRS, which is activated following superoxide exposure (see Table 1-1). The OxyR regulon was found to increase expression of a subset of proteins including antioxidant proteins, such as hydroperoxidase I (*katG*) and alkyl hydroperoxidase reductase (*ahpCF*), which scavenge hydrogen peroxide and broadly limit the damage caused by it [22].

**Table 1-1**. Summary of *E.coli* antioxidant enzymes involved in OxyR, SoxRS, and general stress response (taken from [22]). Genes and their associated functions are separated into the regulons under which they are controlled.

Table 1. Antioxidant activities in *E. coli*[a]

| Gene | Activity |
|---|---|
| *oxyR* regulon | |
| *katG* | Hydroperoxidase I |
| *ahpCF* | Alkyl hydroperoxide reductase |
| *gorA* | Glutathione reductase |
| *grxA* | Glutaredoxin 1 |
| *trxC* | Thioredoxin 2 |
| *fur* | Ferric uptake repressor |
| *dps* | Nonspecific DNA binding protein |
| *oxyS* | Regulatory RNA |
| *agn43* | Outer membrane protein |
| *fhuF* | Ferric reductase |
| *soxRS* regulon (also regulated by MarA and Rob) | |
| *sodA* | Manganese superoxide dismutase |
| *nfo* | Endonuclease IV |
| *zwf* | Glucose-6-phosphate dehydrogenase |
| *fumC* | Fumarase C |
| *acnA* | Aconitase A |
| *tolC* | Outer membrane protein |
| *fur* | Ferric uptake repressor |
| *micF* | RNA regulator of *ompF* |
| *acrAB* | Multidrug efflux pump |
| *nfsA* | Nitroreductase A |
| *fpr* | Ferredoxin/flavodoxin reductase |
| *fldA* | Flavodoxin |
| *fldB* | Flavodoxin |
| *ribA* | GTP cyclohydrolase |
| Other defense activities | |
| *katE* | Hydroperoxidase II |
| *xthA* | Exonuclease III |
| *polA* | DNA polymerase I |
| *recA* | RecA |
| *mutM* (*fpg*) | Formamidopyrimidine glycosylase |
| *nth* | Endonuclease III |
| *mutY* | Adenine glycosylase |
| *msrA* | Methionine sulfoxide reductase |
| *hslO* | Molecular chaperone |
| *sodB* | Superoxide dismutase |
| *sodC* | Copper-zinc superoxide dismutase |
| *iscS* | NifS homolog |
| *iscU* | NifU homolog |

Induction of *fur*, a repressor of iron uptake, also offers global protection by limiting intracellular Fe concentration and therefore the occurrence of Fenton reactions. Additionally, there is induction of glutathione reductase (*gorA*), glutaredoxin 1 (*grzA*), and thioredoxin 2 (*trxC*), all linked to maintaining the thiol-disulfide balance that would be necessary for protein protection. The OxyR protein is turned off by disulfide bond breakage, meaning that its induction of glutaredoxin *grzA* can result in reduction of OxyR's disulfide bonds, thereby providing autoregulation of the regulon [24].

The SoxRS system in *E. coli* responds to intracellular increases in superoxide anions by first activating the SoxR transcription factor, which in turn activates the SoxS regulator [20]. SoxRS upregulates the manganese superoxide dismutase (*sodA*) protein to increase the reducing power of the cell and DNA repair enzyme endonuclease IV (*nfo*) to repair oxidized DNA lesions. Fumarase (*fumC*) and aconitase (*acnA*) are isoenzymes induced by SoxRS due to their resistance to superoxide. There are other proteins involved that either exclude superoxide from entering the cell, or decrease the amount of it being produced inside the cell [22]. While neither the OxyR nor SoxRS regulons are responsible for induction of every gene influenced by hydrogen peroxide or superoxide, they both play central roles in the oxidative stress response of *E. coli*.

The oxidative stress response has also been studied in Archaea. Aerobic archaea have developed many ROS detoxification systems due to their constant environmental

exposure to oxygen [25]. Among these pathways are thioredoxin/glutaredoxin systems, hydroperoxidases, NADH oxidases, and superoxide dismutase [25]. Hydroperoxidases include catalases capable of the decomposition of hydrogen peroxide to $H_2O$ and $O_2$, as well as peroxidases, which catalyzes the breakdown of hydrogen peroxide using other organic reducing agents [25]. Superoxide dismutase decomposes the reactive superoxide radical into the less reactive hydrogen peroxide and $O_2$ and is usually associated with such metal cofactors as Cu, Zn, Mn, Fe, and Ni [25].

Recently, we have focused on understanding the transcriptional response of *H. salinarum* NRC-1 following hydrogen peroxide and superoxide exposure via treatment with paraquat [26]. Following exposure to hydrogen peroxide, genes for the ROS scavenging enzymes PerA (peroxidase), VNG0018H (catalase), and VNG0798H (peroxidase) showed increased transcript levels. Additionally, hydrogen peroxide treatment resulted in upregulation of proteins related to DNA repair and protein turnover, including ribosomal proteins that were not upregulated with superoxide stress. Many metabolic proteins were downregulated with both oxidant treatments, and in particular most proteins containing Fe or iron-sulfur clusters were significantly downregulated [26].

In contrast, after paraquat exposure, *perA* and *sod1* (superoxide dismutase) showed large increases in transcript levels, and modest increases were seen for *VNG0798H*, *VNG0018H*, and *sod2* (accessory superoxide dismutase) [26]. Similar to hydrogen

peroxide exposure, *H. salinarum* NRC-1 upregulated proteins involved in DNA repair and protein turnover and downregulated central metabolism proteins in response to paraquat exposure [26]. It appears that, like *E. coli* and other systems whose ROS response has been studied, *H. salinarum* NRC-1 has global responses to oxidative damage causing molecules like hydrogen peroxide and superoxide. However, it is not known if these enzymatic responses are mediated through some overall regulator like OxyR and SoxRS in *E. coli*. Some proteins may be involved in just one of the responses, while others are regulated by multiple types of stressors.

Additionally, a number of non-enzymatic responses to hydrogen peroxide and paraquat exposure were found. Rhodopsins, retinal binding proteins found in the membrane of *H. salinarum* NRC-1, were determined to be crucial for survival during hydrogen peroxide exposure only [26]. Also during hydrogen peroxide exposure, strains lacking catalase were also found to be deficient in forming proteinaceous gas vesicles necessary for movement up and down in the water column [26]. This was hypothesized to be a protective measure to move away from oxygen sources and therefore limit ROS exposure. In contrast, bleaching of carotenoids in cultures of strains lacking superoxide dismutase provided evidence that these pigments play a role in superoxide scavenging [26]. These findings reflect both the complexity of ROS response in *H. salinarum* NRC-1 and the role of non-enzymatic scavenging in ROS damage avoidance.

*Ionizing radiation and cellular defenses*

As stated previously, the link between desiccation resistance and IR resistance is believed to be through adaptations to oxidative stress that is introduced by both conditions. IR is known to damage cells both directly through ionizing interactions and indirectly through the radiolysis of water producing free radicals [27]. Over 80% of the damage from IR is thought to be the result of ROS production [13]. Radiolysis of water produces hydroxyl radicals, free electrons, and protons (eq. 1). Superoxide production is possible through the interaction between electrons and free $O_2$ (eq. 2):

(1) $H_2O + IR \rightarrow HO^{\bullet} + e^-_{aq}$[hydrated electron] + $H^+$ [proton]

(2) $O_2 + e^- \rightarrow O_2^{\bullet -}$

A variety of other ROS molecules can be generated by subsequent interactions between molecules released through the radiolysis of water and other cellular molecules (Figure 1-1).

For many years, the primary focus of radiation research was on the impact to DNA. It was believed that DNA damage was so severe, either through double strand breaks or extensive damage to individual bases on both strands, that cells were unable to repair their DNA and survive [28]. Subsequent research focused on damage to DNA of IR resistant organisms in the hopes of determining what differentiated them from IR sensitive organisms [29-31]. However, genome sequence and transcriptome analysis of the IR resistant organisms *D. radiodurans*, *Pyrococcus furiosus*, and *H. salinarum* NRC-1 did not reveal any unique DNA repair systems [30-33]. Recent findings indicate DNA double strand breaks from IR are dose-dependant, regardless

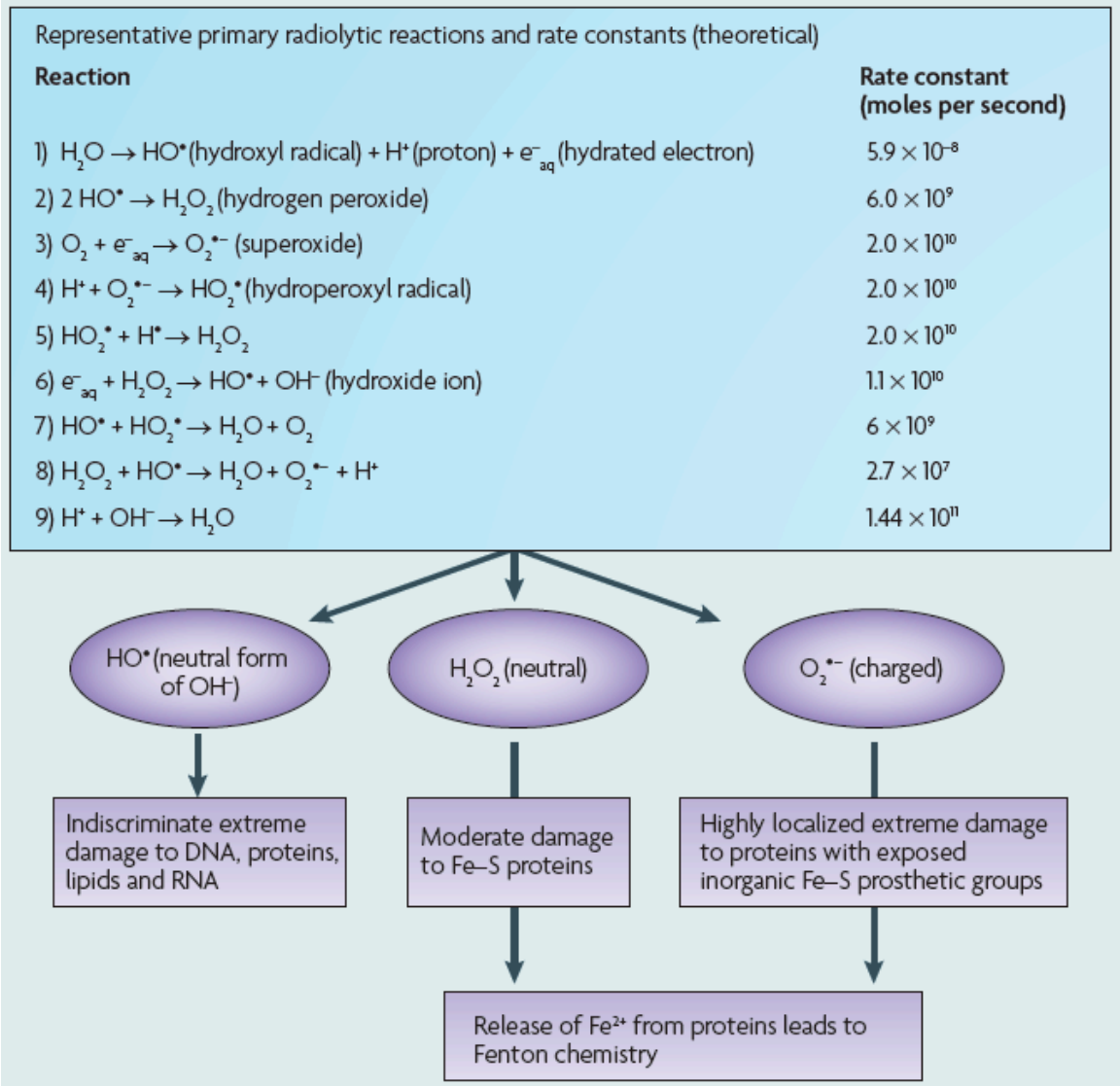


**Figure 1-1**. Theoretical cellular reactions generating a variety of ROS following IR (taken from [34]). The top panel lists expected reactions that result from the radiolysis of water and their rate constants. The bottom panel displays the current model of cellular effects of hydroxyl radicals, hydrogen peroxide, and superoxide generated during IR exposure.

of the host organisms' sensitivity to IR, and do not support the theory that protection of DNA is key to IR resistance [35].

As early as the mid 1990s researchers proposed that proteins were in fact the major target for IR and that protection against protein oxidation – and not DNA damage – was an essential process for survival of IR exposure. The discovery that hydroxyl radicals formed through IR damaged proteins before lipids and DNA indicated that protection of proteins from IR may be critical [36]. In addition, protein radicals that result from ROS exposure are able to propagate damage to other molecules of the cell [37]. Comparison of radiation resistant and sensitive organisms has demonstrated that one key difference in their response to IR is the level of protein oxidation [6, 7]. IR resistant organisms show markedly less protein oxidation in the form of amino acid carbonylation than their IR sensitive counterparts, thus indicating that IR resistant organisms are better able to protect their proteins. This would allow scavenging and repair proteins to perform their roles as soon as the radiation challenge has been removed.

A possible source of this protection from IR is chemical scavengers. Early evidence showed a possible role for Mn in the scavenging of ROS during IR exposure [38]. Complementation of Δ*sod* mutants was possible with the addition of Mn [39, 40] and Mn complexes were shown to limit damage from hydroxyl radicals produced in the presence of hydrogen peroxide and superoxide [41]. *In vitro* assay subsequently demonstrated that Mn, in concert with amino acids, is able to disproportionate $H_2O_2$

to $O_2$ and $H_2O$ [42] and $MnPO_4$ complexes showed superoxide scavenging capabilities [43].

Recent research has shown a correlation between high Mn/Fe ratios and IR resistance in a variety of organisms, including *Deinococcus radiodurans* and *H. salinarum* NRC-1 (Figure 1-2) [34]. It has also been demonstrated that a high intracellular concentration of Mn(II) confers protection on proteins *in vitro* and organisms with high Mn/Fe ratios show less protein damage after irradiation than those organisms with low Mn/Fe ratios [7]. This points to the possibility that Mn(II) could be found in these chemical scavengers that offer protection against IR [34].

*Radiation resistance in the Archaea*

There are several archaeal species that demonstrate IR resistance, including hyperthermophiles and halophiles. *Pyrococcus furiosus* and *Pyrococcus abyssi* are hyperthermophiles that both demonstrate radiation resistance, with $D_{10}$ values, or the IR dose at which 10% of the population survives, of 3.0 and 3.5kGy, respectively [44, 45]. The linear density of double strand breaks after IR exposure has been demonstrated to be the same in the radiation sensitive bacterium *E. coli*, the radiation resistant bacterium *D. radiodurans*, and the radiation resistant archaea *P. furiosus* and *P. abyssi* [35]. *P. furiosus* shows efficient repair of these DNA double strand breaks less than 20 hours after IR [44]. A study of the transcriptomic response of *P. furiosus* to IR demonstrated little evidence of upregulation of DNA repair and ROS detoxification systems after exposure to radiation, indicating that the necessary

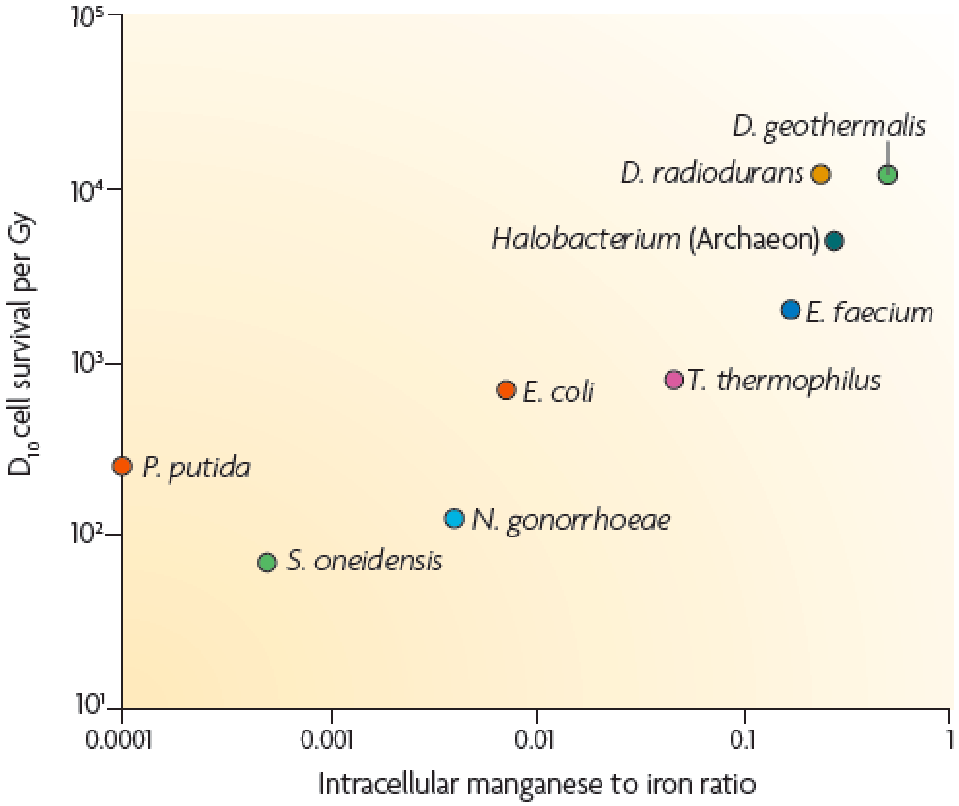


**Figure 1-2**. Relationship between intracellular Mn/Fe ratio and IR resistance (taken from [34]). This demonstrates the correlation between high intracellular Mn/Fe ratios and IR resistance in a number of prokaryotic species, including *H. salinarum* NRC-1.

systems were constitutively expressed [30]. Recent work has isolated additional IR resistant organisms in the class Thermococcus [45, 46]. *Thermococcus gammatolerans* ($D_{10}$ of 6kGy), a hyperthermophilic archaeon isolated from deep-sea hydrothermal chimneys, has demonstrated no loss in cell survival up to 3kGy of IR, and some individuals are capable of surviving after 30kGy of IR [45]. Genomic and proteomic analysis revealed no evidence of duplicated or additional DNA repair genes in *T. gammatolerans* when compared to radiation sensitive Thermococcales organisms [47]. The best-studied archaeal halophile demonstrating IR resistance is *H. salinarum* NRC-1, with a $D_{10}$ value of 5kGy [9]. Its resistance to IR is thought to be a result of its desiccation resistance [3] and its whole genome-transcriptomic response to IR is discussed in the section below [31]. In addition, extremely radiation resistant mutants of *H. salinarum* NRC-1 were selected for through cyclic irradiations; these mutants showed upregulation of a single-stranded DNA-binding protein that may play a role in stabilization of DNA during IR [48].

*Halobacterium salinarum NRC-1 as a model system for oxidative stress studies*

*H. salinarum* NRC-1 is a member of the archaeal kingdom of the *Euryarchaeota* (Figure 1-3). It is halophilic, optimally grown at 4M NaCl and at 42$^{o}$C. The desiccation and high UV radiation encountered in its natural environment suggests that it has protective mechanisms against oxidative stress [2, 3], making it ideal for measuring the effects of various oxidative stressors. *H. salinarum* NRC-1 is useful as a genetic system because its 2.6Mbp genome has been sequenced [33]. It contains one major chromosome and two mini-chromosomes that are all GC-rich. There are

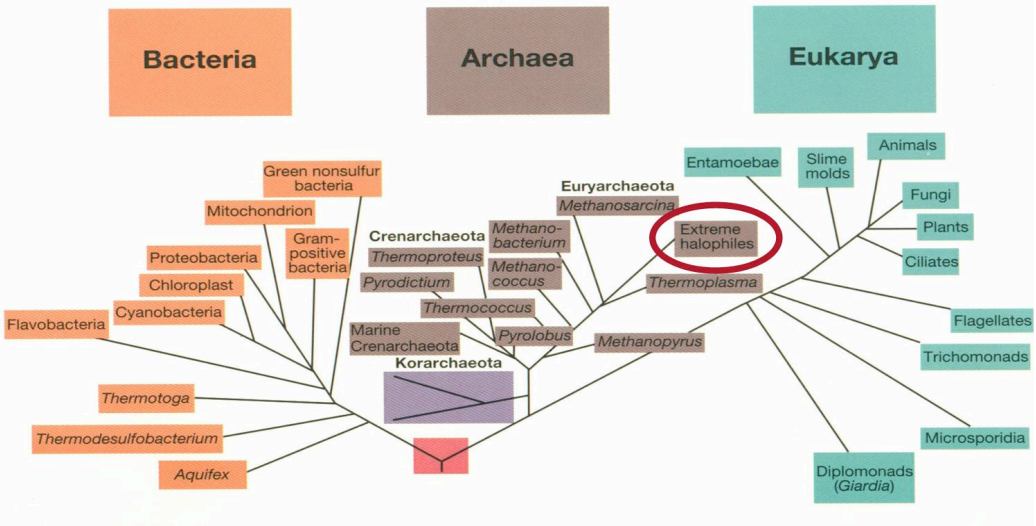


**Figure 1-3.** Phylogenetic tree representing the three domains of life, Bacteria, Archaea, and Eukarya (taken from [49]). The Archaea are divided into three kingdoms, the Crenarchaeota, Euryarchaeota, and Korarchaeota. The extreme halophiles, which include *H. salinarum* NRC-1, are circled in red.

also shuttle-vector systems that make targeted gene deletion possible [50]. Additionally, a whole-genome microarray for detecting mRNA expression has been developed for *H. salinarum* NRC-1 as well as proteomic tools [31].

A systems analysis of *H. salinarum* NRC-1 response to UV radiation, a common challenge in its environment, provided insights into its defense strategies [2]. Among the findings were that only one of the putative photolyases actually played a role in photoreactivation and that there was a general downregulation of proteins not involved in repair, likely to conserve energy for repair [2]. The response of *H. salinarum* NRC-1 has also been assessed in response to desiccation and IR [3]. This study demonstrated that *H. salinarum* NRC-1 is resistant to both desiccation and IR, confirming other studies that showed a link between IR resistance in desiccation resistant organisms [5, 6]. It was found that the membrane pigment bacterioruberin provided protection to *H. salinarum* NRC-1 from IR and that efficient double strand break repair was important for its survival of both desiccation and IR [3]. This efficient repair of DNA double strand breaks can be partly ascribed to the presence of multiple copies of the chromosome in *H. salinarum* NRC-1 [51]. In log phase, *H. salinarum* NRC-1 contains approximately 25 copies of its chromosome, and that drops to approximately 15 copies in stationary phase. This is further supported by the finding that *H. salinarum* NRC-1 is more resistant to IR in log phase as opposed to stationary phase, possibly because there are more genome copies available for homologous recombination during log phase [3].

A systems level analysis of the response of *H. salinarum* NRC-1 to IR found that cells upregulated enzymes involved in DNA repair and oxidative damage repair [31]. This indicates that there may be enzymatic and/or chemical scavengers of ROS necessary to *H. salinarum* NRC-1's resistance to IR. Although we know that *H. salinarum* NRC-1 has a high Mn/Fe ratio similar to other IR resistant organisms, we don't know the role of this chemical scavenger – and possibly others – in the IR resistance of this organism. *H. salinarum* NRC-1 contains high intracellular salts that have demonstrated a protective effect during whole cell irradiations [9], and it has a high Mn/Fe ratio that may implicate Mn in scavenging of ROS generated during ionizing radiation exposure [34]. Understanding the role of enzymatic and chemical scavengers during IR could provide more information as to the reason for *H. salinarum* NRC-1's radiation resistance.

As stated previously, work has been done analyzing *H. salinarum* NRC-1's response to both oxidative stress and IR. *H. salinarum* NRC-1's stress response to hydrogen peroxide and superoxide (via paraquat treatment) resulted in transcriptional changes to proteins involved in detoxification or scavenging of ROS, repair of ROS damage, and maintenance of Fe homeostasis [26]. Peroxidase and catalase were found to play a role in protection against hydrogen peroxide and, to some extent paraquat, while superoxide dismutases were critical only for protection from paraquat treatment [26]. However, no studies have been conducted on the role of those enzymes in the resistance to IR in *H. salinarum* NRC-1, and whether those enzymes are critical in the survival of the organism when exposed to IR.

While it has been demonstrated that ROS treatment has an impact on *H. salinarum* NRC-1 survival [26], the damage induced has not been examined at the molecular level. Due to the differential responses demonstrated after hydrogen peroxide and superoxide stress, it is likely that these two chemical oxidants impact the cells in different ways [26]. In addition, transcriptional changes during IR exposure are also distinct from those experienced in ROS treatment [31]. The cellular role in damage avoidance for enzymes shown to be crucial for survival of oxidant treatment has not been demonstrated.

This work seeks to identify the patterns of damage inflicted on cellular macromolecules of *H. salinarum* NRC-1 as a result of chemical oxidant treatment and IR exposure. DNA damage will be assessed through measurement of specific oxidative DNA lesions using gas chromatography coupled to mass spectrometry (GC/MS) analysis and detection of DNA double strand breaks through pulsed field gel electrophoresis (PFGE). Protein damage will be assessed through two immunodetection assays that survey protein carbonylation. Knockout mutants of enzymes involved in ROS scavenging and DNA repair will be assessed for their roles in protection from oxidative stress. Survival of these mutants and assessment of DNA and protein damage will determine their roles in protection from hydrogen peroxide, superoxide, and IR. Lastly, non-enzymatic scavengers in *H. salinarum* NRC-1 will be examined for their ability to protect DNA and enzyme function from IR. The identities of the non-enzymatic scavengers will be determined through a

chemical analysis. Overall, this work will demonstrate the macromolecular damage introduced by hydrogen peroxide, paraquat, and IR and determine the enzymatic and non-enzymatic damage avoidance mechanisms present in *H. salinarum* NRC-1.

*Research objectives*

The overall focus of this research is to better understand the effects of oxidative damage and IR on *H. salinarum* NRC-1 and to determine cellular mechanisms underlying its response to these challenges. This will elucidate and contrast the damage introduced to cellular molecules by hydrogen peroxide, superoxide, and IR. Additionally, enzymatic and non-enzymatic systems in *H. salinarum* NRC-1 will be evaluated as to their roles in damage avoidance from oxidative stress. My specific aims were:

1. Characterizing the molecular impact of ROS produced by hydrogen peroxide, superoxide, and IR in *H. salinarum* NRC-1. I expected *H. salinarum* NRC-1 to experience different damage profiles from each oxidative stressor due to its unique stress responses to these challenges. Since DNA lesions have thus far been the only measurement of damage induced by ROS in prokaryotes, this work will provide much needed insight into the effects of ROS at a molecular level. Comparison of the cellular impact of different ROS producing conditions was achieved by determining the damage to DNA and proteins after challenging *H. salinarum* NRC-1 with hydrogen peroxide, paraquat (a superoxide producer), and IR.

2. Understanding the cellular role of selected ROS scavenging and DNA repair enzymes of *H. salinarum* NRC-1 in response to oxidative stress. The level of DNA and protein oxidation was determined in knockout mutants of ROS scavenging enzymes (including peroxidase, catalase, and superoxide dismutase) following superoxide, hydrogen peroxide, and IR damage. Comparison of the survival and protein oxidation levels of mutants after IR and chemical oxidant exposure also tested the hypothesis that protein protection is key to IR resistance and survival of oxidative stress. Lastly, the survival of mutants involved in nucleotide excision repair and base excision repair were measured following ROS treatment. This provided a more in-depth understanding of the enzymatic ability of *H. salinarum* NRC-1 to prevent or repair oxidative damage.
3. Determining the non-enzymatic scavengers essential for the survival of *H. salinarum* NRC-1 during IR exposure. Enzyme-free ultrafiltrates consisting of small molecules found in the cytoplasm of *H. salinarum* NRC-1 and the radiation sensitive organisms *E. coli* and *Pseudomonas putida* were examined for their ability to scavenge ROS produced during IR. I expected the ability of *H. salinarum* NRC-1 ultrafiltrate (UF) to be greater in protection of DNA and enzyme function during IR than the other radiation sensitive organisms (*E. coli* and *P. putida*). The chemical composition of the UFs was also probed to determine what differences could account for variations in UF protection capabilities. This work furthered the exploration of non-enzymatic

scavenging of ROS in *H. salinarum* NRC-1 that was begun with the finding that high salt levels protect this organism from IR [9].

This research established the DNA and protein damage profiles of *H. salinarum* NRC-1 in response to hydrogen peroxide, superoxide, and IR. It showed that in *H. salinarum* NRC-1, enzymatic processes for the scavenging of ROS are not critical to IR resistance but that non-enzymatic scavengers of ROS have a central role in protecting cell macromolecules from IR, and proteins in particular. We show a high level of protection for proteins resulting from the accumulation of nucleosides/nucleotides and $MnPO_4$ complexes in the cytoplasm of *H. salinarum* NRC-1. These data provide additional support to the idea that protein protection is an essential key to radiation resistance [7].

# Chapter 2: Cellular damage caused by chemical oxidants and ionizing radiation in *H. salinarum* NRC-1

*Introduction*

Reactive oxygen species (ROS) are produced in aerobic cells during normal cellular respiration. These molecules are capable of damaging such cellular targets as lipids, proteins, and nucleic acids [24]. Damage from ROS has been implicated in a variety of human conditions, including the neurological diseases Alzheimers and Parkinson's [52], aging, and a wide range of cancers [53]. In response to this threat, organisms have developed a number of defense systems and repair mechanisms to combat ROS [22]. Oxidative stress occurs when the levels of ROS overwhelm these defenses and damage begins to accumulate.

**Types of reactive oxygen species**

ROS include such molecules as superoxide, hydrogen peroxide, and hydroxyl radical, all of which can be derived from molecular oxygen. Hydrogen peroxide is a lipid soluble molecule; it can arise either through metabolic activity within the cell or pass through membranes from the extracellular environment [11]. It shows low reactivity, but can be converted into the highly reactive hydroxyl radical through Fenton's reaction [11].

Fenton's reaction:

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^{\bullet}OH + OH^{-}$

Hydroxyl radical has a very short half-life, meaning that it reacts quickly with other molecules in the immediate environment [54]. Since iron (Fe) is often closely associated with DNA and bound to proteins in iron-sulfur clusters (4Fe-4S), these molecules can be targets of oxidative damage [11].

Superoxide, in contrast to hydrogen peroxide, is not membrane soluble and must therefore be produced inside the cell to cause damage [11]. This can arise from normal metabolism or through the introduction of redox-cycling compounds that oxidize redox enzymes and transfer electrons to oxygen to produce superoxide [11]. One example of redox cycling drugs is the herbicide paraquat (*N*,*N*′-dimethyl-4,4′-bipyridinium dichloride) [14]. Superoxide's damage to cellular components relies mainly on the presence of Fe and other transition metals. Superoxide can oxidize iron-sulfur clusters of proteins, thus releasing Fe, which can participate in Fenton's reactions to produce hydroxyl radicals [17]. While Fe is the most likely transition metal to participate in Fenton's reaction, others such as copper and cobalt can also be substituted. In addition, superoxide dismutase can catalyze the dismutation of superoxide to produce hydrogen peroxide and oxygen, and hydrogen peroxide can then damage cells [54].

Ionizing radiation (IR) is known to damage cells both directly through ionizing interactions and indirectly through the hydrolysis of water via the production of free radicals [27]. The most reactive of the radicals produced is the hydroxyl radical, capable of directly damaging DNA, proteins, and lipids via Fenton's reaction. IR can

also produce free electrons that can combine with oxygen to form superoxide radicals [13]. The damage caused by IR will occur throughout the cell wherever gamma rays contact cellular molecules or water. The production of these ROS molecules is more diffuse because IR is deposited throughout the whole cell while hydrogen peroxide must cross the membrane to enter the cell and superoxide is produced primarily around the perimeter of the cell. The high reaction constants of superoxide and hydroxyl radical means they will damage molecules close to their production site while hydrogen peroxide is less reactive and can diffuse further throughout the cell before causing damage [11].

**Damage avoidance and repair systems**

In a response to the ubiquity of ROS and the variety of damage that can result from them, organisms have developed elaborate damage avoidance and damage repair systems. Two of the best-studied oxidative stress response systems are the OxyR and SoxRS regulons in *Escherichi coli*. The OxyR regulon is responsible for the induction of genes following hydrogen peroxide exposure [22]. Antioxidant proteins such as hydroperoxidase I (KatG) and alkyl hydroperoxidase reductase (AhpCF) are upregulated and tasked with eliminating hydrogen peroxide. The Fur protein, a repressor of iron uptake, is upregulated to limit free iron, and thus Fenton's reactions. Additionally, several proteins involved in maintaining the thiol-disulfide balance of proteins are upregulated, increasing protein protection [22].

An increase in superoxide in *E. coli* results in stimulation of the SoxRS system, first activating the SoxR transcription factor, which goes on to activate the SoxS regulator [22]. Proteins induced by SoxRS include superoxide dismutase (SodA), catalyzing the dismutation of superoxide into oxygen and hydrogen peroxide, and fumarase and aconitase, isoenzymes that are resistant to superoxide. Additional genes capable of limiting the amount of superoxide entering the cell or being produced inside the cell are also upregulated [22].

In response to ROS, aerobic archaea developed a number of often overlapping pathways for damage avoidance [25]. These organisms contain many of the same genes central to *E. coli* and other bacteria's oxidative stress responses. These include thioredoxin/gutaredoxin systems necessary for maintenance of the redox levels of cells and ROS scavenging enzymes such as hydroperoxidases, NADH oxidases, and superoxide dismutases [25].

**Detection of oxidized cellular macromolecules**

Several hypotheses exist as to the impact of ROS on cellular biomolecules. Protein damage from ROS is thought to occur mainly through the oxidation of iron-sulfur clusters by hydrogen peroxide or superoxide, thus increasing the free Fe in the cell to participate in Fenton's reactions [11, 17]. Hydrogen peroxide is also involved in metal-catalyzed oxidation of proteins that results in the addition of carbonyl groups to amino acids [12]. Hydrogen peroxide is believed to damage DNA through Fenton's reactions with Fe that is closely associated with the DNA, thus producing hydroxyl

radicals that cause the bulk of the damage [11, 12]. Superoxide's effect on DNA is thought to be more indirect, mainly resulting from the liberation of Fe from iron-sulfur protein clusters, thus increasing the likelihood of Fenton's reaction occurring.

Examination of the effects of ROS on eukaryotic cells have shown the production of a wide variety of modified bases in DNA [55, 56]. In the Archaea, IR exposure of *H. salinarum* NRC-1 resulted in the accumulation of FapyAde (4,6-diamino-5-formamidopyrimidine), FapyGua (2,6-diamino-4-hydroxy-5-formamidopyrimidine), and 8-OH-Gua (8-hydroxyguanine) lesions [9]. The effect of chemical oxidants on production of DNA lesions has not been demonstrated in prokaryotes.

Double stranded breaks of DNA arise from clustered lesions of the DNA [13]. Single strand breaks result from either direct damage from a stressor or due to nicking of the DNA backbone by repair enzymes that are attempting to repair other forms of DNA damage. If single strand breaks to opposite strands of the DNA occurs within approximately 10 base pairs of each other, double strand breaks can result [57]. Double strand breaks have been demonstrated in *H. salinarum* NRC-1 in response to IR [9, 10] and desiccation (DiRuggiero, unpublished), but it is unknown if they are introduced by chemical oxidants.

Oxidation of proteins arises from damage to amino acid side chains, cross-linkages between amino acids, and fragmentation of the protein by breakage of the peptide backbone [58]. Carbonyl derivatives can be formed on lysine, arginine, threonine,

and proline as a result of oxidation [58]. The development of assays able to detect levels of these carbonyl additives to proteins has provided methods for direct detection of protein oxidation [19]. Prokaryotes accumulate protein carbonylation with ionizing radiation [9, 59] and hydrogen peroxide exposure [60, 61]. Paraquat treatment causes an increase in protein carbonylation in eukaryotes [62, 63], however this has not been demonstrated in prokaryotes.

### *H. salinarum* NRC-1 as model organism

*H. salinarum* NRC-1 is a halophilic archaeon that experiences a number of oxidative stressors in its natural environment, such as high UV radiation and desiccation/rehydration cycles [1]. Its resistance to ionizing radiation and desiccation indicate that it is likely well-adapted to oxidative stress [3]. The systemic response of *H. salinarum* NRC-1 to hydrogen peroxide and superoxide stress has demonstrated large-scale responses that are specific to the type of ROS introduced to the cells [26]. Both hydrogen peroxide and paraquat exposure caused upregulation of ROS scavenging enzymes such as superoxide dismutases, catalase and peroxidases [26]. In addition, proteases and proteins involved in DNA repair and maintenance of Fe-S homeostasis were upregulated while central metabolism was downregulated [26]. While many of the transcriptional up- or down-regulations of genes can be ascribed to a generalized environmental stress response, there are subsets of genes affected only by one stress and not the other [26]. During paraquat exposure, there was a move towards anoxic physiology while hydrogen peroxide exposure caused an upregulation of Fe-S oxidoreductase, which contains a common motif involved in OxyR sensing in

*E. coli* [26]. The membrane protein rhodopsin was found to scavenge hydrogen peroxide while the carotenoid pigments scavenged superoxide [26]. Additionally, gas vesicles, which are used for movement up and down in the water column, were produced at higher levels during hydrogen peroxide exposure, possibly as a way for cells to move away from oxygen sources [26]. Therefore, individual ROS have very different impacts on the cell, leading to specific damage-avoidance and repair responses in *H. salinarum* NRC-1.

The transcriptional response of *H. salinarum* NRC-1 to IR showed upregulation of proteases, DNA repair pathways, and nucleotide synthesis [31]. Protection from ROS was likely a concern during IR because central cell metabolism was downregulated while superoxide dismutase and enzymes necessary for maintenance of redox cell levels were upregulated [31]. In addition, damage to specific macromolecules in *H. salinarum* NRC-1 during IR exposure has been measured [9]. IR produces DNA damage in the form of the modified lesions FapyAde, FapyGua, and 8-OH-Gua and through double strand breaks [9]. Proteins of *H. salinarum* NRC-1 are also damaged by IR, resulting in carbonyl modifications [9].

This study focused on the damage of DNA and proteins caused by chemical oxidants in *H. salinarum* NRC-1 to better understand ROS damage at the molecular level. It is hypothesized that hydrogen peroxide, superoxide, and IR will impart unique damage profiles to cellular macromolecules due to the different stress responses elicited by treatment with these ROS treatments [26, 31]. Stress was induced through the

addition of the chemical oxidants hydrogen peroxide and paraquat (a redox cycling drug producing superoxide) or IR and the levels of resulting DNA and protein damage were assessed. Oxidized DNA bases FapyAde and FapyGua detected via gas chromatography coupled to mass spectrometry (GC/MS) and DNA double strand breaks visualized by pulsed field gel electrophoresis (PFGE) were used as evidence of oxidative damage to DNA. Protein oxidation was detected through two immunodetection assays, a western blot and enzyme-linked immuno-adsorbent assay (ELISA). This work is one of the first to evaluate DNA oxidation in prokaryotes as a result of chemical oxidants through GC/MS analysis.

*Materials and Methods*

**Culturing and Growth Conditions**

*Halobacterium salinarum* NRC-1 Δ*ura3* cultures were grown in standard GN101 medium (250g/L NaCl, 20g/L $MgSO_4 \bullet 7H_2O$, 2g/L KCl, 3g/L Na citrate, 10g/L Oxoid brand peptone), pH 7.2, with the addition of 1ml/L trace elements solution (31.5mg/L $FeSO_4 \bullet 7H_2O$, 4.4mg/L $ZnSO_4 \bullet 7H_2O$, 3.3mg/L $MnSO_4 \bullet 7H_2O$, 0.1mg/L $CuSO_4 \bullet 5H_2O$) and 50mg/L uracil and 0.25mg/L 5-flouroorotic acid (5-FOA), final concentration. Cultures were grown at 42$^{o}$C with shaking at 220 rpm in a Gyromax 737 shaker (Amerex Instruments; Lafayette CA) to early log phase ($OD_{600}$=0.4) prior to treatment.

**Oxidative Damage Treatments**

*Peroxide Treatment*

Cells were grown in 25ml cultures of GN101 medium supplemented with uracil to early log phase ($OD_{600}$=0.4) and treated with stock $H_2O_2$ (Sigma; St. Louis, MO) to a final concentration of 25 or 30mM. Cultures were then incubated for 2 hours at 42°C with shaking at 220 rpm in a Gyromax 737 shaker. Cells were pelleted at 8000 x g for 5 minutes, washed with 5ml GN101 + ura, and pelleted again to stop exposure to $H_2O_2$. Cells collected for survival plating and PFGE analysis were processed immediately; cells collected for DNA or protein oxidation analysis were flash frozen in a dry ice/ethanol bath and stored at -80°C until further processing.

*Paraquat Treatment*

Cells were grown in 25ml cultures of GN101 medium supplemented with uracil to early log phase ($OD_{600}$=0.4) and treated with Paraquat (Methyl Viologen; Sigma; St. Louis, MO) to a final concentration of 4 or 10mM. Cultures were then incubated for 2 hours at 42°C with shaking at 220 rpm in a Gyromax 737 shaker. Cells were pelleted at 8000 x g for 5 minutes, washed with 5ml GN101 + ura, and pelleted again to stop exposure to paraquat. Cells collected for survival plating and PFGE analysis were processed immediately; cells collected for DNA or protein oxidation analysis were flash frozen in a dry ice/ethanol bath and stored at -80°C until further processing.

*Gamma irradiation*

Cells were grown in cultures of GN101 medium supplemented with uracil to early log phase ($OD_{600}$=0.40). For cells to be assayed for GC/MS analysis or protein oxidation analysis, 25ml of culture was pelleted with centrifugation at 8000 x g at room temperature for 5 minutes, resuspended in 1ml GN101 + ura, and transferred to a

1.5ml tube to be stored on ice until irradiation. For cells to be assayed for PFGE, 10ml of culture was pelleted at 8000 x g at room temperature for 5 minutes, resuspended in 1ml GN101 + ura, and stored on ice till the irradiation. Irradiations were performed using a $^{60}$Co gamma source (Uniformed Services University of the Health Sciences, Bethesda, MD, dose rate = 3.5kGy/hr) to final doses of 0, 2.5, and 5kGy. Cells collected for PFGE analysis were kept on ice until processing; cells collected for DNA or protein oxidation analysis were flash frozen in a dry ice/ethanol bath and stored at -80$^{o}$C until further processing.

**Survival plating**

Following treatment with oxidative stress, cells were serially diluted in Basal Salt Solution (BSS; 250g/L NaCl, 20g/L $MgSO_4 \bullet 7H_2O$, 2g/L KCl, 3g/L Na citrate) and plated on GN101 medium supplemented with 50mg/L uracil in triplicate. Plates were incubated at 42$^{o}$C for 5-7 days. Survival was calculated as the number of viable cells following treatment divided by the number of viable untreated cells and graphed with standard error bars.

**DNA Oxidation Analysis**

DNA extractions were performed in triplicate as described previously [9]. Briefly, cell pellets were resuspended in BSS at room temperature and transferred to Nalgene bottles. Proteinase K (0.13mg/ml) (Invitrogen; Carlsbad, CA), 2mM desferal (Sigma; St. Louis, MO), and 75ml dd$H_2O$ were added and the cells were incubated at 37$^{o}$C for 1.5 hours. DNA was precipitated with ethanol twice, with extensive washes of 70%

ethanol to eliminate residual salts that would interfere with gas chromatography coupled to mass spectrometry (GC/MS) detection. DNA pellets were stored under 70% ethanol until further analysis. GC/MS with isotope dilution was carried out by Miral Dizdaroglu's group (National Institute of Standards and Technology) as previously described [9]. Briefly, ethanol was removed from DNA pellets and they were dried at room temperature for one hour before being dissolved in water for 24 hours at 4$^{o}$C. The quality and quantity of DNA was determined through absorption spectrophotometry between 200 and 350nm. 50µg aliquots of DNA were dried under vacuum and supplemented with internal standards of isotope-labeled analogues of FapyAde and FapyGua (Cambridge Isotope Laboratories; Cambridge, MA). These were hydrolyzed for one hour with 2µg Fpg, a glycosylase isolated from *E. coli* that is specific for FapyAde and FapyGua and incapable of excision of adenine or guanine from DNA. Fpg was isolated as previously described [64]. After centrifugation, supernatant fractions containing excised FapyAde, FapyGua, and internal standards were lyophilized, trimethylsilylated and analyzed by GC/MS as previously described [64]. Trimethylsilylated FapyAde and FapyGua were identified by monitoring for their characteristic ions during GC/MS and quantified by calculating the integrated areas of the ion signals.

**Pulsed Field Gel Electrophoresis**

Pulsed field gel electrophoresis was performed as described previously [10] in triplicate. Following treatment, the culture $OD_{600}$ was measured and the volume of culture necessary for $2x10^9$ cells was pelleted by centrifugation at 8000 x g for 5

minutes. Cell pellets were resuspended in 500µl BSS; 500µl prewarmed 1.6% InCert agarose solution was added before pouring the mixture into plug molds (BioRad; Hercules, CA). Plugs were incubated overnight at 56°C in 20ml Proteinase K solution (0.25M EDTA, pH 8.0; 1% N-lauroylsarcosine; 0.5mg proteinase K). Plug wash steps to eliminate remaining salts were as follows: 20ml TE Buffer (10mM Tris-HCl, 1mM EDTA, pH 8.0) for 1 hour at 4°C 2x; 20ml 0.5x TE Buffer for 1 hour at 4°C 2x; 20mL 0.5x TE Buffer for 24 hours at 4°C 4x. Plugs were incubated in Pefabloc (Roche; Indianapolis, IN) solution (10mM Tris-HCl, 1mM EDTA, pH 7.0, 1mM Pefabloc) overnight at 37°C, washed in 20ml 2mM Tris-HCl, 5mM EDTA, pH 8.0 for 1 hour at 4°C 3x, and subsequently stored in the same solution at 4°C. Plugs were digested with *XbaI* (New England Biolabs, Ipswich, MA) for 16 hours at 37°C. Following equilibration in 1ml 2mM Tris-HCl, 5mM EDTA, pH 8.0 for 20 minutes at 4°C, plugs were loaded into 1% agarose gels for a CHEF DR-III apparatus (BioRad; Hercules, CA). The gel was run using 0.25x TBE buffer using the following conditions: 6V/cm, 10-60 second switching time, 120 degree included angle, 22 hours, and 12°C. The gel was stained with Ethidium Bromide and imaged with a BioDoc-It Gel Documentation System (UVP; Upland, CA).

**Protein Oxidation Analysis**

*Oxyblot*

Protein analysis was performed as described previously [9]. Briefly, cell pellets were resuspended in 1ml cold 1M salt buffer (50mM potassium phosphate pH 7.0, 1M NaCl, 1% 2-mercaptoethanol) and sonicated for 30 seconds followed by 30 seconds

on ice, repeated three times. Cell lysates were then fractionated by centrifugation at 12000 x g for 30 minutes at 4°C. The soluble proteins in the supernatant were kept on ice and stored at -20°C. Protein concentration was determined using the BioRad Bradford Assay (Hercules, CA). Protein oxidation was detected using the Oxyblot Protein Oxidation Detection Kit (Chemicon/Millipore; Billerca, MA) following the manufacturer's protocol. 20µg of protein sample was derivatized with DNPH (2,4-dinotrophenolhydrazine) to mark the amino acids with carbonyl additions to their side-chains and applied to a 5-20% acrylamide gradient gel (PAGEGel; San Diego, CA) for separation by electrophoresis at 150V, 50mAmps, for 2.5 hours. The proteins were then transferred to a PVDF membrane (Millipore; Billerca, MA) via Western transfer at 25V, 30mAmps, for 16 hours. Immunodetection was performed using primary (anti-DNP) and secondary (HRP-conjugated) antibodies provided by the manufacturer, followed by incubation in SuperSignal West Pico Chemiluminescent Substrate (Pierce; Rockford, IL) and imaged using Hyperfilm ECL (Amersham Biosciences; Piscataway, NJ) exposed for 30 seconds to 4 minutes.

*OxyElisa*

Protein analysis was performed in triplicate. Preparation of protein samples was carried out as for oxyblot analysis (see above). Protein oxidation was assessed using the OxiSelect Protein Carbonyl ELISA Kit (Cell Biolabs; San Diego, CA) and the manufacturer's protocol. Cell lysates were diluted to 10µg/ml of protein in 1xPBS and 1µg of protein was added to each well in a 96-well protein binding plate. Protein carbonyl BSA standards were also prepared ranging from 0µg/ml to 7.5µg/ml and 1µg of each was added to the wells of the plate and incubated at 4°C overnight.

Three washes of 250µl 1xPBS were performed followed by incubation with 4µg of DNPH for 45 minutes at room temperature to derivatize the carbonyl additions to the proteins. Five washes of 250µl 1xPBS/Ethanol (1:1, v/v) and by two washes of 250µl 1xPBS were performed, followed by incubation with blocking buffer for 2 hours at room temperature with shaking. Wells were washed three times with 250µl 1x wash buffer and incubated with the anti-DNP antibody for 1 hour at room temperature with shaking. Three washes with 250µl 1x wash buffer were again performed and the samples were incubated with HRP conjugated secondary antibody for 1 hour at room temperature with shaking. Following five washes with 250µl 1x Wash Buffer, 100µl Substrate Solution was added to the wells and incubated for 2-5 minutes and the reaction was stopped with the addition of 100µl Stop Solution. Absorbance of each well was then read in a Power Wave 200 Microplate Spectrophotometer (Bio-tek Instruments; Winooski, VT) at 450 nm. A standard curve was constructed using the samples supplied with the kit and was used to determine the protein carbonylation levels of the oxidant-treated samples.

*Results*

Based on previous work with wild type *H. salinarum* NRC-1 and this study, we established that the 80% survival of the *H. salinarum* NRC-1 Δ*ura3* strain was at 25mM hydrogen peroxide and 4 mM paraquat [26]. The *H. salinarum* NRC-1 Δ*ura3* strain (described as *H. salinarum* NRC-1 below) lacks uracil biosynthesis capabilities and was used as a control because the mutants studied in Chapter 3 were constructed in this background. We first characterized the damage caused by oxidant treatment to

DNA and proteins in *H. salinarum* NRC-1 (Figure 2-1). Damage to DNA was determined by measuring the amount of FapyGua lesions, an oxidized form of guanine, in DNA after treatment with hydrogen peroxide or paraquat using GC/MS analysis (Figure 2-1 A). The average of three replicates showed FapyGua lesions were more numerous in cells exposed to hydrogen peroxide than paraquat. While the increase in FapyGua lesions with paraquat treatment was statistically significant ($p = .027$) there were only about 0.2 more FapyGua lesions per million bases when the background levels were subtracted out. In cells treated with hydrogen peroxide, there were about 1 more lesion per million DNA bases. DNA damage was also measured by the detection of double strand breaks. Cells were treated with chemical oxidants and genomic DNA was immobilized in PFGE plugs that were digested with *XbaI* restriction enzyme. The resulting DNA fragments were separated by pulsed-field gel electrophoresis. DNA with no strand breakage resulted in distinct bands on the agarose gel while fragmented chromosomal DNA appeared as a smear. The same banding pattern was found for untreated and treated cells, indicating that there was no genome fragmentation, even at higher doses of paraquat (10mM) or hydrogen peroxide (30mM) (Figure 2-1 B). Protein oxidation in the form of carbonyl residues detected by the OxyELISA assay showed less than a one-fold increase in hydrogen peroxide treated cells when compared to untreated cells (Figure 2-1 C). A very slight increase in carbonylation was found in paraquat treated cells over untreated cells, yet it was not large enough to be statistically significant.

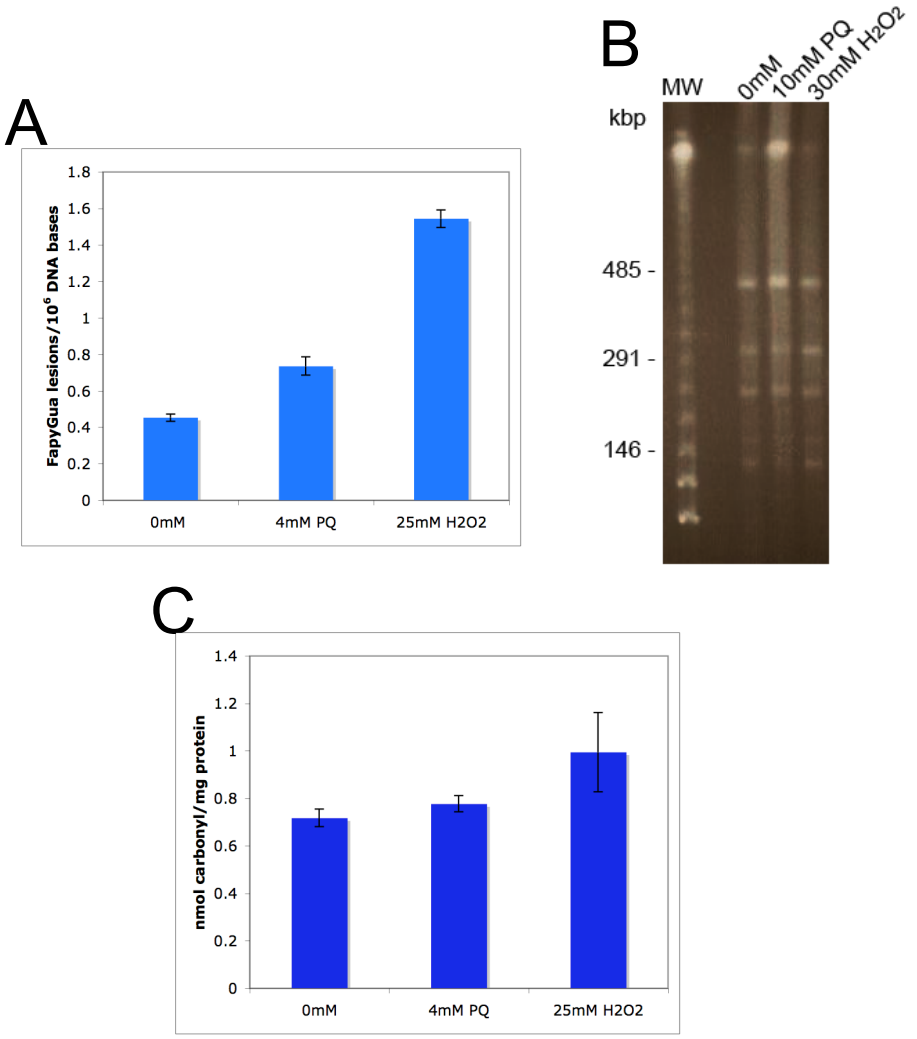


**Figure 2-1**. Oxidative lesions of *H. salinarum* NRC-1 Δ*ura3* cells untreated (0mM) and exposed to paraquat (PQ) and hydrogen peroxide ($H_2O_2$). (A) FapyGua DNA lesions were measured by GC/MS with isotope dilution in cells treated with 4mM PQ and 25mM $H_2O_2$. (B) DNA double strand breaks were measured by PFGE in cells treated with 10mM PQ and 30mM $H_2O_2$. (C) Protein carbonylation was measured by the OxyElisa assay in cells treated with 4mM PQ and 25mM $H_2O_2$. MW = molecular weight marker; kbp = kilobase pairs. 0mM are untreated controls. Data shown are the averages of at least 3 replicates and uncertainties are represented as standard error.

We also measured DNA and protein damage in *H. salinarum* NRC-1 Δ*ura3* exposed to IR. 80% survival for *H. salinarum* NRC-1 exposed to IR is 2.5kGy, which is similar to the results from wild type *H. salinarium* NRC-1 [3]. FapyGua and FapyAde lesions roughly double with exposure to 2.5kGy IR when compared to untreated samples (Figure 2-2 A). The FapyGua lesions increased by about 3.5 lesions per million DNA bases, and this increase was greater than what we found in cells treated with chemical oxidants (Figure 2-1 A). In contrast to the results from chemical oxidant treatment, IR does cause DNA double strand breaks to the chromosome of *H. salinarum* NRC-1 (Figure 2-3 B). Lastly, protein oxidation increases close to four-fold in cells treated with 2.5kGy ionizing radiation when compared to untreated cells (Figure 2-2 C), which is a significantly higher increase than that of cells treated with paraquat or hydrogen peroxide (Figure 2-1 C).

Since our chemical oxidant $D_{80}$ (dose corresponding to 80% survival) represents two hours of treatment, we decided to focus upon what damage was occurring earlier in the treatment. *H. salinarum* NRC-1 showed consistent levels of 80% survival over the two hours of oxidant treatments (Figure 2-3). DNA damage was assessed over the two hour time period. FapyGua lesions were more numerous in the hydrogen peroxide treated cells than the paraquat treated cells when compared to the untreated control, and the level of lesions stayed consistent over the two hour time period (Figure 2-4 A). In addition, genome fragmentation was not evident during any of the time points for hydrogen peroxide and paraquat treatments (Figure 2-4 B). There also

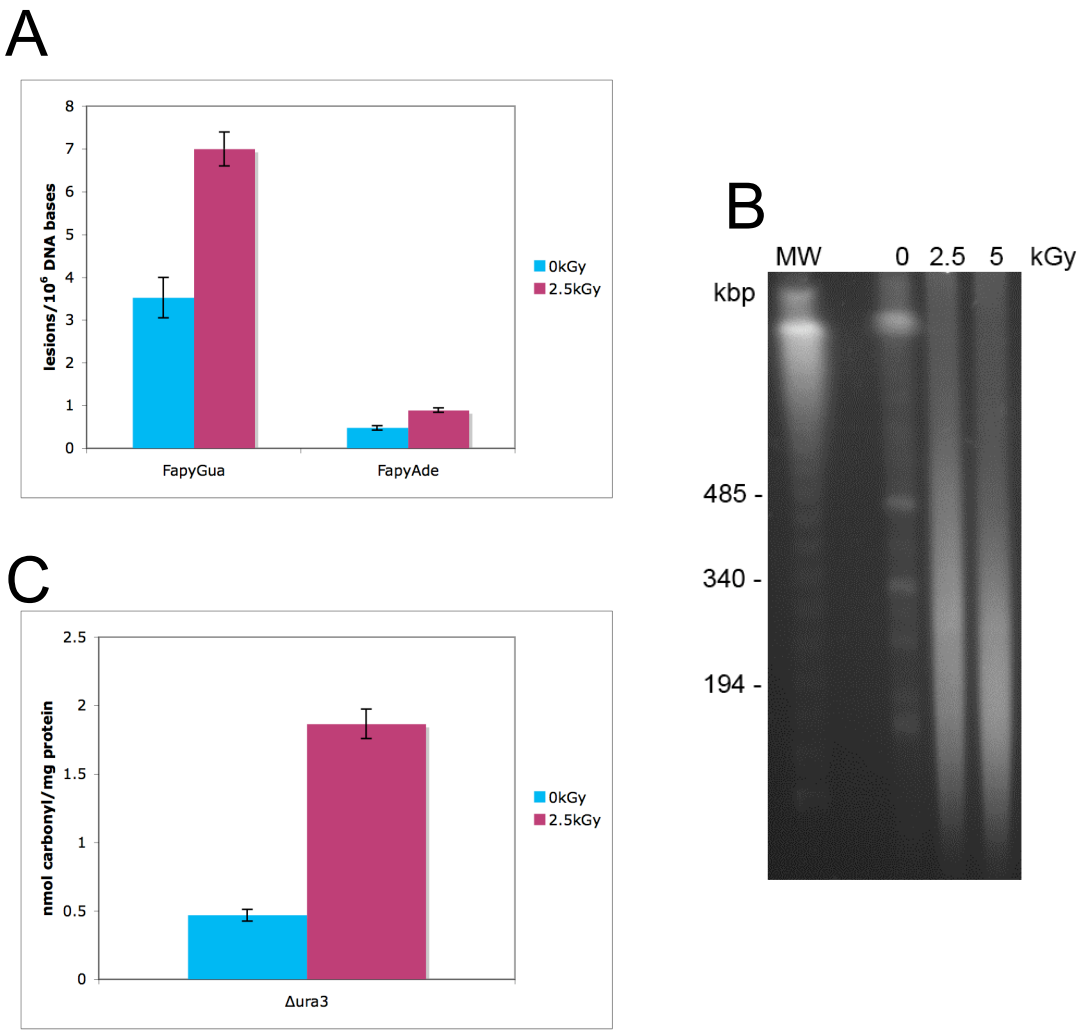


**Figure 2-2**. Oxidative lesions of *H. salinarum* NRC-1 Δ*ura3* cells untreated (0kGy) and treated with ionizing radiation. (A) FapyGua and FapyAde DNA lesions were measured by GC/MS with isotope dilution in cells treated with 2.5kGy ionizing radiation. (B) DNA double strand breaks were measured by PFGE in cells treated with 2.5 and 5kGy ionizing radiation. (C) Protein carbonylation was measured by the OxyElisa assay in cells treated with 2.5kGy ionizing radiation. MW = molecular weight marker; kbp = kilobase pairs. Data shown are the average of at least 3 trials with uncertainty represented as standard error.

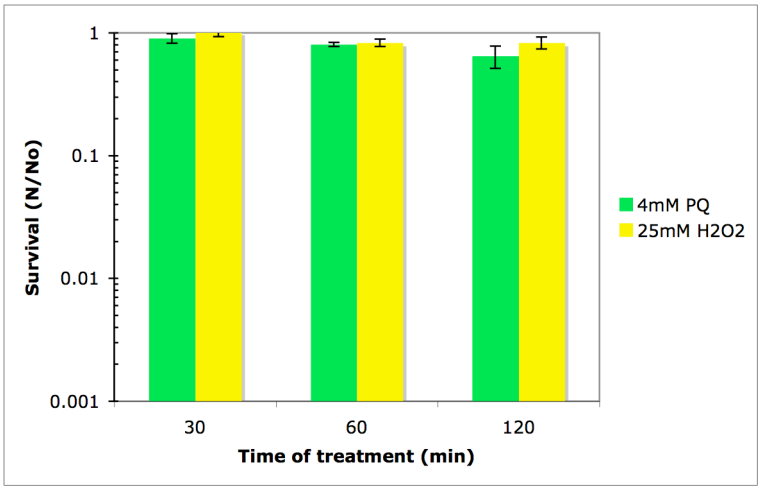


**Figure 2-3**. Survival of *H. salinarum* NRC-1 Δ*ura3* over 2 hours of treatment with 4mM paraquat (PQ) and 25mM hydrogen peroxide ($H_2O_2$). Survival was calculated as the average ratio (N/No) of surviving colony forming units from treated (N) compared to untreated (No) cultures. Data shown are the average of at least 3 replicates and uncertainty is represented as standard error.

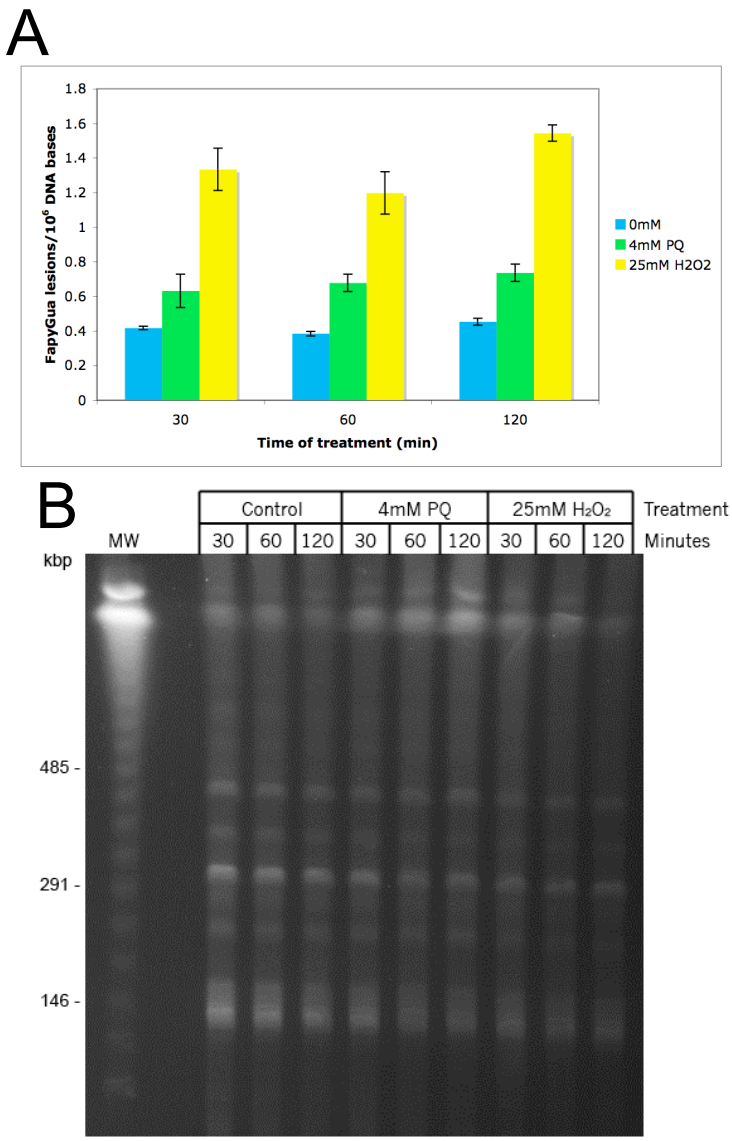


**Figure 2-4**. DNA lesions of *H. salinarum* NRC-1 Δ*ura3* cells treated with paraquat (4mM PQ) and hydrogen peroxide (25mM $H_2O_2$) for up to two hours. (A) FapyGua DNA lesions were measured by GC/MS with isotope dilution in cells treated with PQ and $H_2O_2$ for 30, 60, and 120 minutes. (B) DNA double strand breaks were measured by PFGE in cells treated with PQ and $H_2O_2$ for 30, 60, and 120 minutes. Data shown are the average of at least 3 replicates and uncertainty is represented as standard error. MW = molecular weight; kbp = kilobase pairs.

did not appear to be an increase in carbonylation of peptides between the 30-minute and 120-minute time points (Figure 2-5 A and B).

*Discussion*

Our experimental setup for exposure to chemical oxidants was initially established to analyze changes in gene expression during two hours of exposure to chemical oxidants [26]. However, it was important to assess cell survival and cellular damage occurring during that 2-hour period to validate our comparisons with IR. Survival of *H. salinarum* NRC-1 was consistent at 80% for all of the time points. In addition, there was no significant change in the levels of DNA lesions, double strand breaks, or protein oxidation for any of the time points. There are two possible explanations for this: (1) cells began repairing damage shortly after exposure and levels of damage and repair were roughly equal throughout the time course or (2) most damage occured early, by the 30-minute time point and subsequent damage was not detectable.

There are several pieces of evidence that support the first hypothesis. Our finding of fairly steady levels of survival, DNA damage, and protein oxidation indicate damage had begun by the 30 minute time point and the amount of subsequent damage and repair are roughly equal, as seen by a lack of accumulation or drop-off in damage. Recovery after IR exposure under optimal conditions showed repair of FapyGua lesions occuring under two hours and turn-over of oxidized proteins was complete by eight hours [9]. In addition, during paraquat and hydrogen peroxide exposure, proteases and most genes involved in scavenging ROS and repair are upregulated by

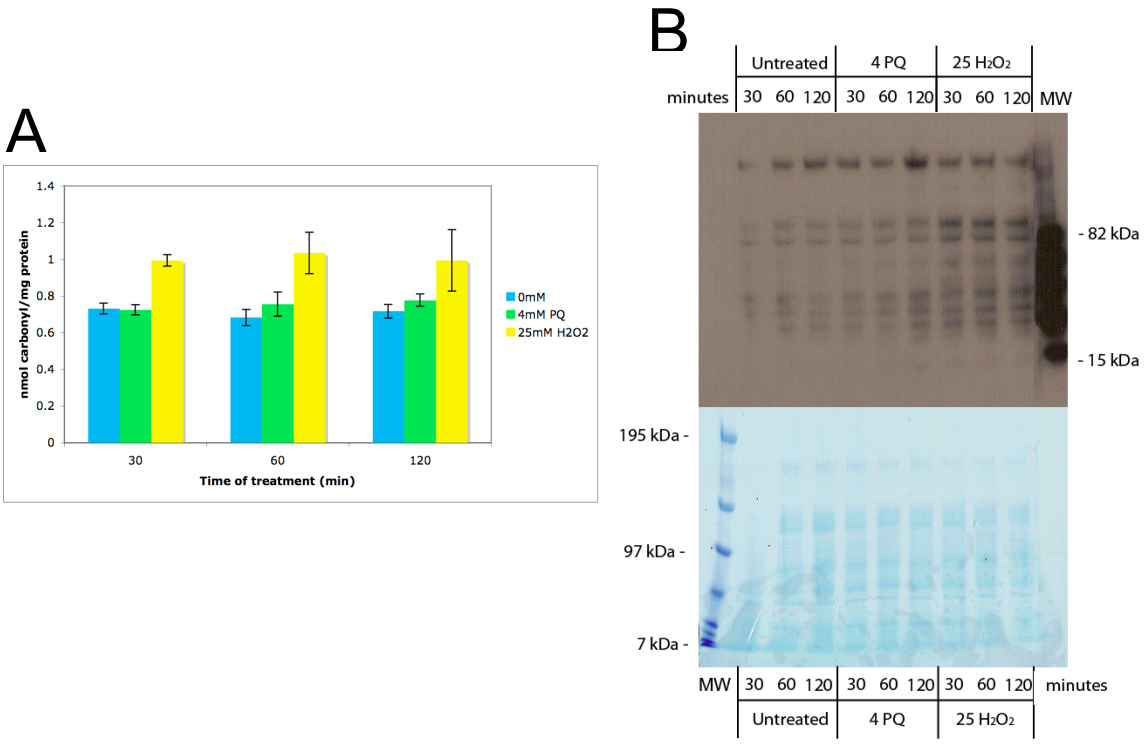


**Figure 2-5**. Protein oxidation of *H. salinarum* NRC-1 Δ*ura3* cells treated with paraquat (4mM PQ) and hydrogen peroxide (25mM $H_2O_2$) for up to two hours. (A) Protein carbonylation was measured by the OxyElisa assay in cells treated with 4mM PQ and 25mM $H_2O_2$ for 30, 60, and 120 minutes. (B) Protein carbonylation was measured by the Oxyblot assay in cells treated with 4mM PQ and 25mM $H_2O_2$ for 30, 60, and 120 minutes. Top: western blot showing the level of protein carbonylation. Bottom: Western blot stained with coomassie blue showing total protein loaded on the gel. Data shown are the average of at least 3 replicates and uncertainty is represented as standard error. MW = molecular weight; kDa = kilodaltons.

20 minutes after beginning treatment [26]. This would indicate that the damage avoidance and damage repair responses of *H. salinarum* NRC-1 are swift and could be alleviating the damage introduced by oxidants even as they are introducing more damage. The decomposition of paraquat has been studied in the environment due to its use as an herbicide and was found to heavily depend upon UV light exposure [65]. Exposure to sunlight for 48 hours resulted in over 60% photodegradation of paraquat [65], so it is unlikely that two hours of artificial light exposure in this experiment would result in significant loss of paraquat toxicity. Hydrogen peroxide may be less stable in solution because it is more likely to decompose in low concentration solution and GN101 medium contains trace amounts of transition metals that can catalyze hydrogen peroxide's decomposition. Overall, there seems to be more evidence that the two-hour time point represented both the damage to cells and repair response of *H. salinarum* NRC-1, however the decomposition of the oxidants and their subsequent loss in toxicity cannot be ruled out.

This work also allowed for comparison of the damage to cellular macromolecules caused by chemical oxidants and IR in *H. salinarum* NRC-1. One of the major differences found between the stresses was the presence of DNA double strand breakage after IR but not hydrogen peroxide or superoxide treatment. The lack of DNA double strand breaks with hydrogen peroxide and paraquat may be due to the localization of damage on the DNA molecule. DNA double strand breaks are thought to arise from single strand breaks within 10 to 20 base pairs of each other [57, 66], indicating that damage must be clustered, which is what is typically found with IR

[66]. 80% of the damage caused by ionizing radiation is in the form of ROS molecules produced via the radiolysis of water [13]. When this occurs near DNA, hydroxyl radicals produced would cause heavy damage to a limited area due to their high rate constant [12]. Some of those lesions resulted in backbone breakage whereas others introduce oxidative damage to bases and sugars of the DNA. In this later case, attempted repair by DNA repair enzymes results in backbone breakage and, when lesions are clustered, the introduction of DNA double strand breaks. This would explain the DNA fragmentation that we observed with the *H. salinarum* NRC-1 chromosome following IR. An IR dose of 2.5 kGy introduced around 65 DNA double strand breaks and those strand breaks were repaired in less than 8 hours [9].

In contrast, hydrogen peroxide was introduced externally to the cells and diffused through the cellular membrane and into the cytoplasm. DNA damage from hydrogen peroxide is usually the result of its interaction with Fe bound to the DNA to produce hydroxyl radicals through Fenton's reaction [12]. While hydroxyl radicals will damage the DNA in the area of the bound Fe, it is unlikely to result in significant double strand breakage. The production of superoxide from paraquat treatment is strictly localize to the membrane due to its reliance on redox enzymes for generation of superoxide [11]. Superoxide damages the DNA indirectly through the release of Fe from iron-sulfur protein clusters to increase the likelihood of Fenton's reactions occurring [11], meaning that it is unlikely to produce clustered damage to DNA like what is seen from IR.

DNA damage in *H.salinarum* NRC-1 was also measured in terms of oxidized bases produced as the result of paraquat, hydrogen peroxide, and IR treatment. There are a wide variety of DNA oxidized lesions, however a limited number of them can be accurately measured [67]. Two examples are FapyGua and FapyAde, both of which were previously detected in wild type *H. salinarum* NRC-1 following IR [9]. The levels of FapyGua and FapyAde in DNA increased with IR dosage, there were more FapyGua lesions observed than FapyAde lesions, and the damaged bases produced by the 2.5kGy dose were repaired by two hours post-irradiation [9]. Our data presented here with *H. salinarum* NRC-1 Δ*ura3* confirmed those results with a one-fold increase in both FapyGua and FapyAde lesions in the irradiated versus non-irradiated cells.

We found that after subtracting the background level of lesions in the untreated cells from the treated cells, the largest increase in DNA lesions occurred in cells treated with IR. We found that FapyGua lesions increased by approximately 3.5 lesions per million DNA bases after IR while chemical oxidants introduced less than 1 lesion per million bases. In concert with our finding that IR introduces DNA double strand breaks yet hydrogen peroxide and paraquat do not, this indicates that IR damages DNA more than chemical oxidants in *H. salinarum* NRC-1. The introduction of different levels of hydroxyl radicals with each treatment may account for this finding. IR introduces hydroxyl radicals through the radiolysis of water that can impart damage to DNA bases, and IR is likely deposited throughout the entire cell [13]. With hydrogen peroxide treatment, there is production of hydroxyl radicals due to

Fenton's reactions, and this often occurs in close association with DNA due to Fe bound to it [12]. However, hydrogen peroxide must diffuse throughout the cell to deposit damage and it is unclear the extent to which this occurs. Superoxide produced by paraquat does not damage DNA directly, but rather indirectly by liberating free Fe to participate in Fenton's reactions or the formation of other ROS molecules [11]. The variation in levels of oxidized DNA lesions after ROS treatment, despite 80% survival for all conditions, also indicates that oxidation of DNA is not correlated with survival. This was also reported for IR when radiation sensitive and resistant organisms experience roughly the same amount of DNA double strand breaks after IR exposure [59]. This indicates that survival of IR is not due to prevention of DNA damage, but rather its efficient repair [59].

Irreversible oxidation of amino acids can occur as a result of IR or metal-catalyzed oxidation [24]. Damage to the amino acids arginine, lysine, proline, and threonine can result in irreversible carbonylation of their side chains [19]. As a result, number of methods have been developed to measure levels of protein carbonylation, and it has become an accepted measure of overall protein oxidation in cells [19]. The two methods utilized for detecting protein oxidation in this study measure the levels of protein carbonylation in cells that have been damaged with oxidative stress.

Protein oxidation showed a three-fold increase following IR exposure compared to untreated cells. In contrast, when compared to untreated cells, hydrogen peroxide treatment caused a slight increase in protein oxidation and there was no significant

change in protein oxidation following paraquat treatment. IR can damage cellular molecules in two ways, by direct ionization, producing free radicals that can combine with oxygen to form peroxyl radicals [13], or via radiolysis of water producing mainly hydroxyl radicals, protons, and electrons that can combine with $O_2$ to form superoxide [13]. Superoxide is relatively long lived and can interact with protein iron-sulfur clusters to release $Fe^{2+}$ and increase the likelihood of Fenton's reaction occurring [11]. IR's production of superoxide is distributed throughout the cell, meaning it can interact with a wide variety of proteins, while superoxide production by paraquat occurs mainly around the perimeter. Hydrogen peroxide, while better able to diffuse throughout the cell, has a lower rate constant for interaction with iron-sulfur clusters than superoxide [11]. In conclusion, our results illustrate the fact that the location of ROS production heavily influences the damage experienced by proteins.

We found that IR produced a three-fold protein carbonylation increase than exposure to hydrogen peroxide and paraquat whereas the level of survival of the cells for each of these treatments was similar. These findings are in contrast to experiments with bacteria showing that the level of protein carbonylation was negatively correlated with survival [7]. It is likely that chemical oxidants are causing severe stress to the cells in other ways not detected by the analytical methods used in this study. Paraquat produces superoxide by taking electrons from redox proteins involved in cellular respiration [11], possibly interfering with metabolism and energy production which would result in decreased cell fitness and survival. Additionally, IR produces

ROS inside the cell while paraquat and hydrogen peroxide are introduced extracellularly and must cross the cell membrane to cause damage. Hydrogen peroxide exposure in *E. coli* generates higher levels of lipid peroxidation [68]. Additionally, paraquat causes membrane damage in epithelial cells that is hypothesized to be the result of increased extracellular hydrogen peroxide levels that attack the membranes [69]. IR, in contrast, causes little lipid peroxidation in mouse myeloma cells [36]. Therefore, lipid peroxidation may be more of a challenge in cells treated with chemical oxidants rather than IR. Further understanding of the level of lipid oxidation in *H. salinarum* NRC-1 could demonstrate the validity of this claim.

Another argument supporting the idea that damage to specific metabolic pathways might result from exposure to paraquat was the observation by Kaur and colleagues [26] that despite the similar impact on survival of *H. salinarum* NRC-1, the oxidative stress response caused by 4mM paraquat seemed to be more significant than that caused by 25mM hydrogen peroxide [26]. The sub-inhibitory dose of 0.25mM paraquat even resulted in a different stress response than 4mM paraquat [26]. At lower concentrations, paraquat treatment elicited a faster stress response than 4mM paraquat and caused upregulation of ribosomal genes to increase protein turnover, one of the superoxide dismutases, and Fe-S oxidoreductases that are not induced at higher concentrations of paraquat [26]. Furthermore, there is evidence in other organisms that the cellular response to redox-cycling drugs, including paraquat, may not be due to superoxide exposure [11]. In *Pseudomonas aeruginosa* under anaerobic conditions, redox-cycling drugs were able to elicit a response from the SoxR regulon

in the absence of superoxide [70]. This indicates that paraquat may be stimulating the cells in ways that are not exclusively related to superoxide production.

This study demonstrates that there are different damage profiles resulting from hydrogen peroxide, paraquat, and IR exposure in *H. salinarum* NRC-1. We believe that differences in molecular damage imparted by each ROS treatment are due to the localization of ROS production in cells. IR damage is more evenly distributed throughout the cells, leading to more DNA double strand breaks and protein oxidation, while paraquat produces superoxide at the perimeter of the cells and hydrogen peroxide must diffuse across the cell membrane, resulting in less evenly distributed ROS from chemical oxidants. In addition, these treatments corresponded to the same level of survival of *H. salinarum* NRC-1, and different levels of DNA and protein damage, indicating that survival during chemical oxidant treatment is not correlated with protein or DNA damage. This work provides a clearer picture of what the effects of different ROS producers are on cellular macromolecules and provides some insight into the time necessary for chemical oxidants to introduce damage to *H. salinarum* NRC-1 cells.

# Chapter 3: Roles of ROS scavenging enzymes and DNA repair systems in oxidant and ionizing radiation challenges of *H. salinarum* NRC-1

*Introduction*

**Reactive oxygen species scavenging enzymes**

Reactive oxygen species (ROS) are molecules derived from oxygen, including superoxide, hydrogen peroxide, and hydroxyl radicals, that can damage cell proteins, DNA, and lipids [24]. They are introduced to organisms both endogenously, typically from enzymatic reactions relating to metabolism, and exogenously through chemical reactions in the environment or production by other organisms [11]. In response, cells have developed a number of damage-avoidance or damage repair systems to deal with these stressors. When the level of ROS overwhelms these defense systems, the result is oxidative stress to the cells. A number of enzymes have been discovered in aerobic and some anaerobic organisms to play important roles in the scavenging of ROS in response to oxidative stress [11]. Among key enzymes are superoxide dismutase, catalase/peroxidase systems, and methionine sulfoxide reductase.

Superoxide dismutase (SOD) disproportionates superoxide to $O_2$ and hydrogen peroxide via the following reactions:

(1) $M^{3+} + O_2^{\bullet-} \rightarrow M^{2+} + O_2$

(2) $M^{2+} + 2H^+ + O_2^{\bullet-} \rightarrow M^{3+} + H_2O_2$

where M stands for the metal cofactor [71]. Based on metal cofactors, SODs can be separated into two major groups. Cu/Zn- SODs are mainly found in the cytoplasm of eukaryotes and the periplasm of prokaryotes while Fe- or Mn- SODs are found in prokaryotes and the mitochondria of eukaryotes [71]. Ni-SODs have also been discovered, but so far only identified in *Streptomyces* species [71, 72]. The Mn-SOD of *H. salinarum* NRC-1 is closely related to Fe-SODs of other archaea and suggests a common evolutionary origin [71]. In *E.coli*, Fe-SOD is constitutively expressed while Mn-SOD is upregulated under the SoxRS regulon that responds to superoxide stress [73]. The huge abundance of SOD in *E. coli* cells is coupled with its extremely efficient breakdown of superoxide; its steady-state concentration, close to 0.1nM indicates that limiting superoxide exposure is key to the cells [11].

Hydroperoxidases are enzymes capable of neutralizing hydrogen peroxide and include catalases and peroxidases. Peroxidases detoxify hydrogen peroxide via the following reaction:

$RH_2 + H_2O_2 \rightarrow R + 2H_2O$

where R is an organic reducing agent. They contain a reducible heme group [25]. At low hydrogen peroxide levels, the primary detoxification process in *E. coli* occurs via the activity of the alkyl hydroperoxide reductase AhpCF [11]. When the concentration of hydrogen peroxide is over 20μM, the activity of AhpCF reaches its limit, the OxyR regulon is induced, and the KatG catalase is upregulated to increase hydrogen peroxide scavenging [11].

Catalase functions by dismutating hydrogen peroxide as follows:

$H_2O_2 + H_2O_2 \rightarrow O_2 + 2H_2O$

The catalase gene in *E. coli*, *katG*, is upregulated under hydrogen peroxide stress by the OxyS regulon and its protein becomes the primary hydrogen peroxide scavenger under high-stress conditions [22]. Since catalase does not rely upon outside reducing agents like peroxidase does, its turnover rate is not limited by the availability of those molecules [11]. Its high rate of reaction and turnover means that *E.coli* can maintain intracellular hydrogen peroxide concentrations up to an order of magnitude lower than the extracellular concentration [11].

Oxidation of the amino acid methionine can result in the formation of methionine sulfoxide (MetO), which is one of the few ROS-derived protein lesions that can be repaired [19]. Conversion of the lesion back to methionine can be carried out by methionine sulfur reductases (Msr), thioredoxin, and thioredoxin reductase [74]. MetO is produced as one of two enantiomers, and MsrA is capable of reducing the S-MetO isomer while MsrB reduces the R-MetO isomer [74, 75]. Genes encoding these enzymes have been identified in organisms from all three domains [75]. The reduction of MetO by MsrA is demonstrated in the following reaction:

$MetO + TR(SH)_2 \rightarrow Met + TR(S\text{-}S) + H_2O$

where thioredoxin ($TR(SH)_2$) is oxidized [19]. Thioredoxin reductase reduces TR(S-S) back to thioredoxin ($TR(SH)_2$), resulting in a cyclic oxidation/reduction of methionine and having antioxidant effects [19].

*Halobacterium salinarum* NRC-1 contains two superoxide dismutase genes, *sod1* and *sod2*, two peroxidases, *perA* and *VNG0798H*, a putative catalase, *VNG0018H*, and a methionine sulfoxide reductase gene, *msrA*. In addition, peroxiredoxins and enzymes responsible for production of secondary radical scavengers such as carotenoids and glycerol have been identified [26]. In a whole-genomic transcriptomic analysis focusing on the response of *H. salinarum* NRC-1 to hydrogen peroxide and superoxide stress, we found that the superoxide dismutases, peroxidases, and catalase were upregulated under both stressors [26]. Further analysis using gene deletion mutants provided more evidence for their cellular roles. While we found that both superoxide dismutases were induced during superoxide stress, the characterization of deletion mutants revealed that Sod1 is the major scavenger while Sod2 played a more accessory role. In contrast, neither of those proteins were critical for survival during hydrogen peroxide stress [26]. Under hydrogen peroxide stress, the characterization of gene deletion mutants showed that PerA and VNG0798H were critical for survival while VNG0018H played a lesser role. Both *perA* and *VNG0018H* deletion mutants were less able to survive paraquat exposure than the control strain, showing that they each offer some cross protection under superoxide stress [26]. An earlier study on *H. salinarum* NRC-1's response to IR found ROS scavenging enzymes, including superoxide dismutase, to be upregulated during IR exposure [31].

**Nucleotide and base excision repair pathways**

In addition to scavenging of ROS in organisms, repair of damage caused by oxidation is also critical. The repair of DNA damage is of utmost importance for cells; if left

unrepaired, DNA lesions will result in mutagenesis that leads to cell death or is propagated to future generations. In addition, DNA lesions can cause replication blocks and attempted repair can produce double strand breaks, both potentially leading to cell death. There are a wide variety of oxidative DNA lesions, including lesions to the sugar and bases, and phosphate backbone breakage, however only a small number of those lesions can be quantified experimentally [67]. FapyAde and FapyGua are two examples of oxidized lesions that can be measured, and both can be repaired by the base excision repair pathway (BER) [76]. Another common DNA repair pathway is the nucleotide excision repair (NER), which is capable of recognizing and removing bulky lesions in DNA [76].

BER is a process that is conserved in both prokaryotes and eukaryotes. It is one of the most commonly used DNA repair pathways that functions through recognition of altered and damaged bases in DNA [76]. The first step of the BER pathway is recognition of oxidized purines and pyrimidines by DNA glycosylases that are target-specific [77]. Although these glycosylases only recognize a narrow range of damage, cells typically produce a variety of enzymes to maximize the amount of damages that can be detected and repaired [78]. Glycosylases that are monofunctional remove individual bases, leaving an intact apurinic or apyrimidic (AP) site, and rely upon an AP endonuclease to cleave the DNA backbone on the 5' side of the AP site [76]. Bifunctional glycosylases both remove the damaged base and have AP lyase activity that cleaves the DNA backbone 3' of the AP site [76]. The resulting gaps at the AP

site are filled by DNA polymerase using the complementary strand as a template and DNA ligase that seals the phosphate backbone, resulting in repaired DNA [77].

In Archaea, understanding of the BER pathway has mostly been accomplished using hyperthermophiles [79, 80]. BLAST sequence alignment of *H. salinarum* NRC-1's genome has revealed three putative glycosylase genes: *ogg*, the 8-oxo-Gua glycosylase and *nthA1* and *nthA2*, both endonuclease III glycosylases. Both Ogg and Nth are bifunctional and therefore display the AP lyase ability to cleave DNA 3' of the AP site [76]. The glycosylase Ogg has shown a high specificity for removal of 8-oxoGuanine, FapyGuanine, and FapyAdenine [76, 81] while Nth in yeast and *E. coli* is a able to remove multiple pyrimidine-derived lesions [76, 82].

The NER pathway removes bulky oxidized lesions from the DNA. In *E.coli*, NER functions through the UvrABC system [83]. UvrA and UvrB form a complex that scans DNA looking for distortions to the double helix, which are the result of bulky, mismatched lesions. When one is encountered, UvrB binds to the DNA base, UvrA disengages and is replaced by UvrC. This UvrB/UvrC complex then excises DNA several bases up- and down-stream of the lesion, resulting in a short single-stranded region of DNA. UvrD binds to the open 3' end of DNA, polymerase resynthesizes the region, and ligase seals the phosphate backbone, yielding repaired DNA [83].

Eukaryotic NER repair is performed by a more varied set of genes than in Bacteria, however they perform roughly the same functions [84]. Most Archaea contain

Eukaryotic-like NER repair genes, however only the mesophilic methanogens and halophiles also contain genes homologous to the UvrABC system in Bacteria [76]. The canonical bacterial UvrABC system is thought to be present in only selected archaea as the result of a recent lateral gene transfer event [76]. In *H. salinarum* NRC-1, gene deletion mutants for *uvrA*, *uvrB*, and *uvrC* displayed a loss of survival after UV exposure and dark repair, indicating that this pathway functions in the repair of cyclobutane dimers and 6,4-photoproducts introduced by UV [85]. Photoreactivation repair of these lesions was previously shown, indicating that *H. salinarum* NRC-1 has developed efficient and overlapping mechanisms for limiting damage from UV radiation that is a major challenge in its environment [2].

### *H. salinarum* NRC-1 as a model organism

*H. salinarum* NRC-1 is an excellent model organism for a genetic approach to understanding the role of specific enzymes and repair systems in survival during oxidative stress. It routinely experiences oxidative stress in its natural aerobic environment with high levels of solar radiation and desiccation [2, 3], suggesting that it possesses robust systems for ROS damage avoidance. The 2.6 Mbp genome of *H. salinarum* NRC-1 has been sequenced [33] and protocols for engineering gene deletion mutants have been developed [50].

Transcriptomic studies characterizing *H. salinarum* NRC-1 responses to various environmental conditions, including UV radiation [2], ionizing radiation (IR) [31], and chemical oxidants [26] have been reported. In addition, characterization of gene

deletion mutants have provided insight into the roles of ROS scavenging enzymes [26] and the function of the NER pathway in repair of UV-induced DNA damage [85].

Our focus was to better understand the roles of ROS scavenging and NER/BER enzymes in *H. salinarum* NRC-1's oxidative stress response. Oxidative stress was induced through treatment with hydrogen peroxide, paraquat, and IR. These three treatments were selected because they differ in the effectors of the oxidative stress; hydrogen peroxide is lipid soluble and able to generate hydroxyl radicals via Fenton's reaction [11], paraquat is a redox cycling compound that generates superoxide radicals once it enters the cell [14], and superoxide increases intracellular Fe levels through damage to iron-sulfur clusters in proteins [17] and it is broken down by superoxide dismutase to produce oxygen and hydrogen peroxide [54]. IR damages cells through ionizing reactions and primarily through the radiolysis of water and subsequent production of ROS such as hydroxyl radicals and superoxide [13].

Using a genetic approach, we characterized the role of ROS scavenging enzymes in the protection of *H. salinarum* NRC-1 against oxidative stress. Levels of DNA and protein damage from treatments by chemical oxidants and IR were analyzed in deletion mutants of ROS scavenging enzymes to determine if these enzymes played critical roles in the protection of those macromolecules. A high level of protein protection against oxidation has been proposed as a key feature for survival in IR resistant organisms [6, 7]. Here, with the analysis of ROS mutant survival and their protein oxidation levels, we tested if this hypothesis extends to the survival of

chemical oxidant treatment. In addition, gene deletion mutants of the NER/BER pathways were assessed for their survival during oxidant and IR exposure to determine if these repair pathways are essential for maintenance of genome integrity during oxidative stress.

*Materials and Methods*

**Culturing and Growth Conditions**

*Halobacterium salinarium* sp. strain NRC-1 cultures were grown in standard GN101 medium (250g/L NaCl, 20g/L $MgSO_4 \bullet 7H_2O$, 2g/L KCl, 3g/L Na citrate, 10g/L Oxoid brand peptone), pH 7.2 with the addition of 1ml/L trace elements solution (31.5mg/L $FeSO_4 \bullet 7H_2O$, 4.4mg/L $ZnSO_4 \bullet 7H_2O$, 3.3mg/L $MnSO_4 \bullet 7H_2O$, 0.1mg/L $CuSO_4 \bullet 5H_2O$). When specified, cultures were supplemented with 50mg/L uracil and 0.25mg/L 5-flouroorotic acid (5-FOA), final concentrations. Cultures were grown at $42^oC$ with shaking at 220 rpm in a Gyromax 737 shaker (Amerex Instruments; Lafayette CA) to early log phase ($OD_{600}$=0.4) prior to treatment.

**Construction of Gene Deletion Strains**

Gene deletions of Δ*ogg*, Δ*nthA1*, Δ*nthA2*, and Δ*ogg*Δ*nthA2* were constructed as described in Peck et al. [50] and Kish et al. [10]. Briefly, uracil drop-out medium was used to select for uracil autotrophy following transformation of Δ*ura3* with the plasmid pNBK07 containing the knockout gene construct and *ura3* gene marker for uracil biosynthesis. Medium containing 5-FOA was then used to select for recombinants that had gained the deletion construct gene but lost the plasmid

containing the *ura3* gene. Recombinants were screened using PCR. GN101 medium was supplemented with uracil when growing all mutants. Gene deletions of Δ*sod1/2*, Δ*perA*, Δ*msrA*, Δ*VNG0798H*, and Δ*VNG0018H* were provided by Dr. Baliga (Institute for Systems Biology) and Δ*uvrA* was provided by Dr. Crowley (Assumption College). Strains used in this study are summarized in Table 3-1.

**Oxidative Damage Treatments**

*Peroxide Treatment*

Cells were grown in 25ml cultures of GN101 medium supplemented with 50 mg/L uracil to early log phase ($OD_{600}$=0.4) and treated with stock $H_2O_2$ (Sigma; St. Louis, MO) to a final concentration of 25 or 30mM. Cultures were then incubated for 2 hours at 42$^{o}$C with shaking at 220 rpm in a Gyromax 737 shaker. Cells were pelleted at 8000 x g for 5 minutes, washed with 5ml GN101 + ura, and pelleted again to stop exposure to $H_2O_2$. Cells collected for survival plating were processed immediately; cells collected for DNA or protein oxidation analysis were flash frozen in a dry ice/ethanol bath and stored at -80$^{o}$C until further processing.

*Paraquat Treatment*

Cells were grown in 5ml cultures of GN101 medium supplemented with 50mg/L uracil to early log phase ($OD_{600}$=0.4) and treated with Paraquat (Methyl Viologen; Sigma; St. Louis, MO) to a final concentration of 4 or 10mM. Cultures were then incubated for 2 hours at 42$^{o}$C with shaking at 220 rpm in a Gyromax 737 shaker. Cells were pelleted at 8000 x g for 5 minutes, washed with 5ml GN101 + ura, and pelleted again to stop exposure to paraquat. Cells collected for survival plating were

**Table 3-1**. *H. salinarum* NRC-1 strains used in this study.

| | **Strain name** | **Genotype** | **Cellular function of encoded protein** |
|---|---|---|---|
| **ROS Mutants** | *Δura3* | *Δura3* | uracil biosynthesis* |
| | *Δsod1/2* | *Δura3Δsod1Δsod2* | superoxide dismutases |
| | *ΔperA* | *Δura3ΔperA* | peroxidase |
| | *ΔmsrA* | *Δura3ΔmsrA* | methionine sulfoxide reductase |
| | *ΔVNG0798H* | *Δura3ΔVNG0798H* | peroxidase |
| | *ΔVNG0018H* | *Δura3ΔVNG0018H* | catalase |
| | | | |
| **BER Mutants** | *Δogg* | *Δura3Δogg* | 8-oxoguanine glycosylase |
| | *ΔnthA1* | *Δura3ΔnthA1* | endonuclease III |
| | *ΔnthA2* | *Δura3ΔnthA2* | endonuclease III |
| | *ΔoggΔnthA2* | *Δura3ΔoggΔnthA2* | |
| | | | |
| **NER Mutant** | *ΔuvrA* | *Δura3ΔuvrA* | Nucleotide excision repair pathway* |

*cellular funtion experimentally verified. ROS: reactive oxygen species; BER: base excision repair; NER: nuceotide excision repair.

processed immediately; cells collected for DNA or protein oxidation analysis were flash frozen in a dry ice/ethanol bath and stored at -80$^{o}$C until further processing.

*Gamma irradiation*

Cells were grown in cultures of GN101 medium supplemented with 50mg/L uracil to early log phase ($OD_{600}$=0.40). For survival assays, 1ml aliquots of the cultures were transferred to 1.5ml tubes and stored on ice until irradiation. For GC/MS analysis or protein oxidation analysis, 25ml of culture was pelleted at 8000 x g at room temperature for 5 minutes, resuspended in 1ml GN101 + ura, and transferred to a 1.5ml tube to be stored on ice till irradiation. Irradiations were performed using a $^{60}$Co gamma source (Uniformed Services University of the Health Sciences, Bethesda, MD, dose rate = 3.5kGy/hr) to final doses of 0, 2.5, and 5 kGy. Samples were kept on ice until further processing was performed.

**Survival Testing**

Following treatment with oxidative stress, cells were serially diluted in Basal Salt Solution (BSS; 250g/L NaCl, 20g/L $MgSO_4$•$7H_2O$, 2g/L KCl, 3g/L Na citrate) and plated on GN101 medium supplemented with 50mg/L uracil in triplicate. Plates were incubated at 42$^{o}$C for 5-7 days. Survival was calculated as the number of viable cells following treatment divided by the number of viable untreated cells and graphed with standard error bars. Survival testing of the ROS mutants was performed by Courtney Busch.

**DNA Oxidation Analysis**

DNA extractions were performed in triplicate as described previously [9]. Briefly, cell pellets were resuspended in BSS at room temperature and transferred to Nalgene bottles. Proteinase K (0.13mg/ml) (Invitrogen; Carlsbad, CA), 2mM desferal (Sigma; St. Louis, MO), and 75ml dd$H_2O$ were added and the cells were incubated at 37$^oC$ for 1.5 hours. DNA was precipitated with ethanol twice, with extensive washes of 70% ethanol to eliminate residual salts that would interfere with gas chromatography coupled to mass spectroscopy (GC/MS) detection. DNA pellets were stored under 70% ethanol until further analysis. GC/MS with isotope dilution was carried out by Miral Dizdaroglu's group (National Institute of Standards and Technology) as previously described [9]. Briefly, ethanol was removed from DNA pellets and they were dried at room temperature for one hour before being dissolved in water for 24 hours at 4$^oC$. The quality and quantity of DNA was determined through absorption spectrophotometry between 200 and 350nm. 50µg aliquots of DNA were dried under vacuum and supplemented with internal standards of isotope-labeled analogues of FapyAde and FapyGua (Cambridge Isotope Laboratories; Cambridge, MA). These were hydrolyzed for one hour with 2µg Fpg, a glycosylase isolated from *E. coli* that is specific for FapyAde and FapyGua and incapable of excision of adenine or guanine from DNA. Fpg was isolated as previously described [64]. After centrifugation, supernatant fractions containing excised FapyAde, FapyGua, and internal standards were lyophilized, trimethylsilylated and analyzed by GC/MS as previously described [64]. Trimethylsilylated FapyAde and FapyGua were identified by monitoring for their characteristic ions during GC/MS and quantified by calculating the integrated areas of the ion signals.

**Protein Oxidation Analysis**

*Oxyblot*

Protein analysis was performed as described previously [9]. Briefly, cell pellets were resuspended in 1ml cold 1M salt buffer (50mM potassium phosphate pH 7.0, 1M NaCl, 1% 2-mercaptoethanol) and sonicated for 30 seconds followed by 30 seconds on ice, repeated three times. Cell lysates were then fractionated by centrifugation at 12000 x g for 30 minutes at 4$^{o}$C. The soluble proteins in the supernatant were kept on ice and stored at -20$^{o}$C. Protein concentration was determined using the BioRad Bradford Assay (Hercules, CA). Protein oxidation was detected using the Oxyblot Protein Oxidation Detection Kit (Chemicon/Millipore; Billerca, MA) following the manufacturer's protocol. 20µg of protein sample was derivatized with DNPH to mark the amino acids with added protein carbonyl groups and applied to a 5-20% acrylamide gradient gel (PAGEGel; San Diego, CA) for separation by electrophoresis at 150V, 50mAmps, for 2.5 hours. The proteins were then transferred to a PVDF membrane (Millipore; Billerca, MA) via Western transfer at 25V, 30mAmps, for 16 hours. Immunodetection was performed using primary (anti-DNP) and secondary (HRP conjugated) antibodies provided by the manufacturer, followed by incubation in SuperSignal West Pico Chemiluminescent Substrate (Pierce; Rockford, IL) and imaged using Hyperfilm ECL (Amersham Biosciences; Piscataway, NJ) exposed for 30 seconds to 4 minutes.

*OxyElisa*

Protein analysis was performed in triplicate. Preparation of protein samples was carried out as for oxyblot analysis (see above). Protein oxidation was assessed using the OxiSelect Protein Carbonyl ELISA Kit (Cell Biolabs; San Diego, CA) using the manufacturer's protocol. Cell lysates were diluted to 10µg/ml of protein in 1xPBS and 1µg of protein was added to each well in a 96-well protein binding plate. Protein carbonyl BSA standards were also prepared ranging from 0µg/ml to 7.5µg/ml and 1µg of each was added to the wells of the plate and incubated at 4$^{o}$C overnight. Three washes of 250µl 1xPBS were performed followed by incubation with 4µg of DNPH for 45 minutes at room temperature to derivatize the carbonyl additions to the proteins. Five washes of 250µl 1xPBS/Ethanol (1:1, v/v) followed by two washes of 250µl 1xPBS were performed, followed by incubation with blocking buffer for 2 hours at room temperature with shaking. Wells were washed three times with 250µl 1x wash buffer and incubated with the anti-DNP antibody for 1 hour at room temperature with shaking. Three washes with 250µl 1x wash buffer were again performed and the samples were incubated with HRP conjugated secondary antibody for 1 hour at room temperature with shaking. Following five washes with 250µl 1x Wash Buffer, 100µl Substrate Solution was added to the wells and incubated for 2-5 minutes and the reaction was stopped with the addition of 100µl Stop Solution. Absorbance of each well was then read in a Power Wave 200 Microplate Spectrophotometer (Bio-tek Instruments; Winooski, VT) at 450 nm. A standard curve was constructed using the samples supplied with the kit and was used to determine the protein carbonylation levels of the oxidant-treated samples.

**Oxidant pretreatment experiment**

A schematic representing the oxidant pretreatment experiment is in Figure 3-1. *H. salinarum* NRC-1 Δ*ura3* cultures were grown in GN101 supplemented with 50mg/L uracil to early log phase ($OD_{600}$=0.4) and split into three separate cultures for pretreatment. Pretreatments of 0mM oxidant control, 2mM paraquat, and 10mM $H_2O_2$ were administered with the cells incubated at 42$^{o}$C for 30 minutes with shaking at 220 rpm. Aliquots of 5ml were taken from each culture, pelleted at 8000 x g for 5 minutes at room temperature, and washed with 5ml GN101 supplemented with uracil, and pelleted again. Cell pellets were resuspended in 5ml GN101 + ura and 1ml was reserved for each IR dose (0, 2.5, and 5 kGy). Cell suspensions were left at room temperature for 1 hour to allow them to produce any necessary ROS scavenging enzymes and then kept on ice. Irradiations were performed using a $^{60}$Co gamma source (National Institute of Standards and Technology, Gaithersburg, MD) to final doses of 0, 2.5, and 5 kGy. Samples were kept on ice until survival plating was performed as previously described.

*Results*

The survival of *H. salinarum* NRC-1 ROS scavenging mutants after paraquat and hydrogen peroxide treatment was previously determined (Figure 3-2) [26]. Here we showed that the Δ*ura3* background strain used in our mutant construction exhibited approximately 80% survival after 4mM paraquat and 25mM hydrogen peroxide treatments. Following 4mM paraquat treatment, the mutant strain Δ*sod1/2* showed a significant decrease in survival, close to two orders of magnitude, when compared to

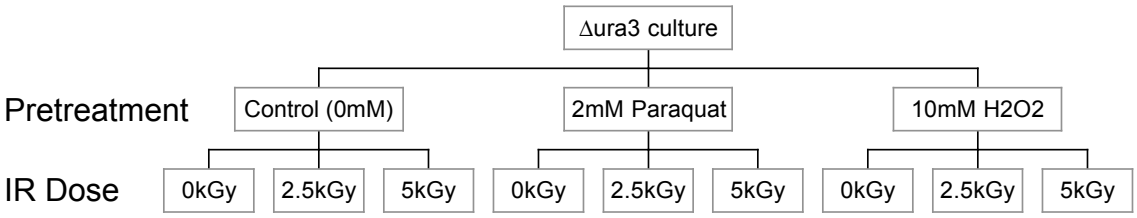


**Figure 3-1**. Experimental design for *H. salinarum* NRC-1 Δ*ura3* survival of IR following oxidant pretreatment.

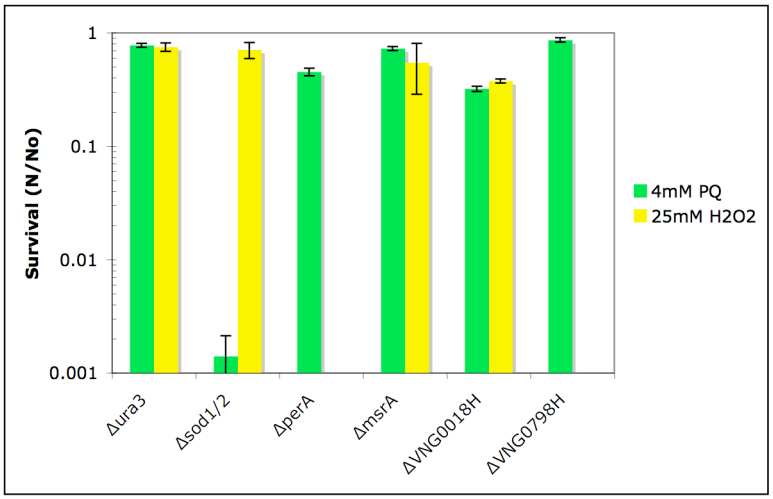


**Figure 3-2**. Survival of *H. salinarum* NRC-1 mutants following treatment with hydrogen peroxide and paraquat (adapted from [26]). The strains Δ*ura3* (background strain), Δ*sod1/2*, Δ*perA*, Δ*msrA*, Δ*VNG0018H*, and Δ*VNG0798H* were exposed to 25mM H2O2 and 4mM Paraquat and their survival was determined by a plating assay. These represent results from at least 3 independent cultures and uncertainty is presented as standard error. This work was performed by Courtney Busch and published in part in [26].

the control strain Δ*ura3*; in contrast, the strains Δ*VNG0018H* and Δ*perA* showed 32% and 45% survival, respectively. Exposure to 25mM hydrogen peroxide resulted in complete killing of Δ*perA*, a severe loss in survival for Δ*VNG0798H* with regard to the control strain Δ*ura3*, and only a 38% survival for Δ*VNG0018H*. Surprisingly, treatment with IR at 2.5kGy (approximately 80% survival) and 5kGy did not result in a significant decrease in survival for any of the mutants when compared to the Δ*ura3* control strain (Figure 3-3).

The survival of *H. salinarum* NRC-1 NER/BER mutants was also tested with chemical oxidants. Neither the Δ*uvrA* mutant (NER) nor the Δ*ogg*, Δ*nthA1*, Δ*nthA2*, and Δ*ogg*/Δ*nthA2* mutants (BER) showed any additional decrease in survival with 10mM paraquat or 30mM hydrogen peroxide when compared to the control strain Δ*ura3* (Figure 3-4 A). Survival was tested at higher oxidant concentrations because no additional decrease in survival was found with 4mM paraquat or 25mM hydrogen peroxide (data not shown). Representative mutants from the NER pathway (Δ*uvrA*) and BER pathway (Δ*ogg*) displayed the same level of survival as the control strain following 2.5kGy of IR (Figure 3-4 B).

The level of DNA damage in ROS mutants was surveyed to determine if there was a correlation between DNA lesions and survival to oxidative stress. FapyGua lesions were measured in DNA from *H. salinarum* NRC-1 mutant and control strains treated with paraquat, hydrogen peroxide, and IR at doses resulting in 80% survival of the control strain (Figure 3-5). The only mutants showing greater increases in FapyGua

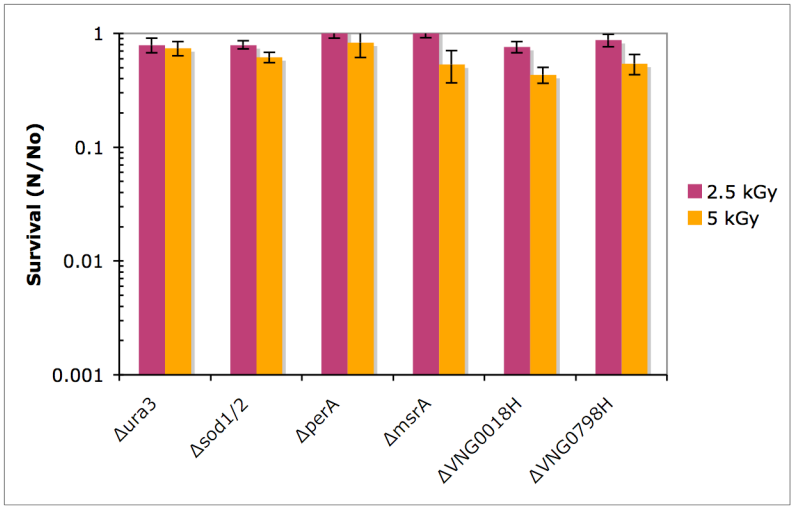


**Figure 3-3**. Survival of *H. salinarum* NRC-1 mutants following treatment with IR. The strains Δ*ura3* (background strain), Δ*sod1/2*, Δ*perA*, Δ*msrA*, Δ*VNG0798H*, and Δ*VNG0018H* were exposed to 2.5 and 5kGy IR and their survival was determined by a plating assay. These represent results from at least 3 independent cultures and uncertainty is presented as standard error.

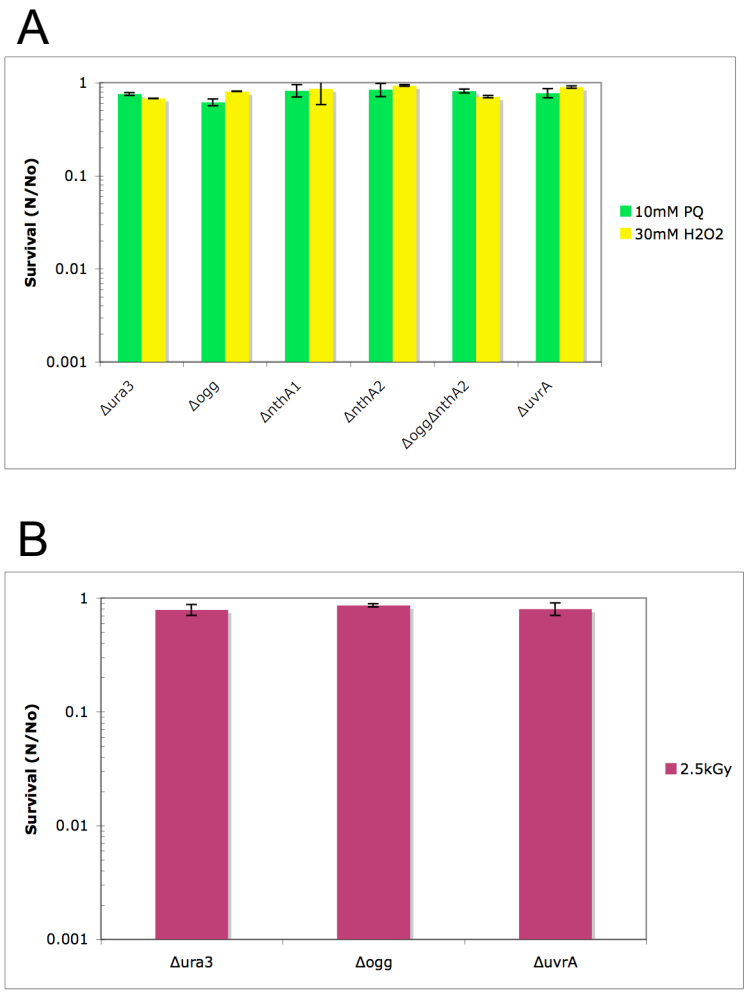


**Figure 3-4.** Survival of *H. salinarum* NRC-1 mutants following chemical oxidant treatment and IR. (A) The strains Δ*ura3* (background strain), Δ*ogg*, Δ*nthA1*, Δ*nthA2*, Δ*ogg*Δ*nthA2*, and Δ*uvrA* were exposed to 10mM Paraquat and 30mM hydrogen peroxide and their survival was determined by a plating assay. (B) The strains Δ*ura3* (background strain), Δ*ogg*, and Δ*uvrA* were exposed to 2.5kGy IR and their survival was determined by a plating assay. These represent results from at least 3 independent cultures and uncertainty is presented as standard error.

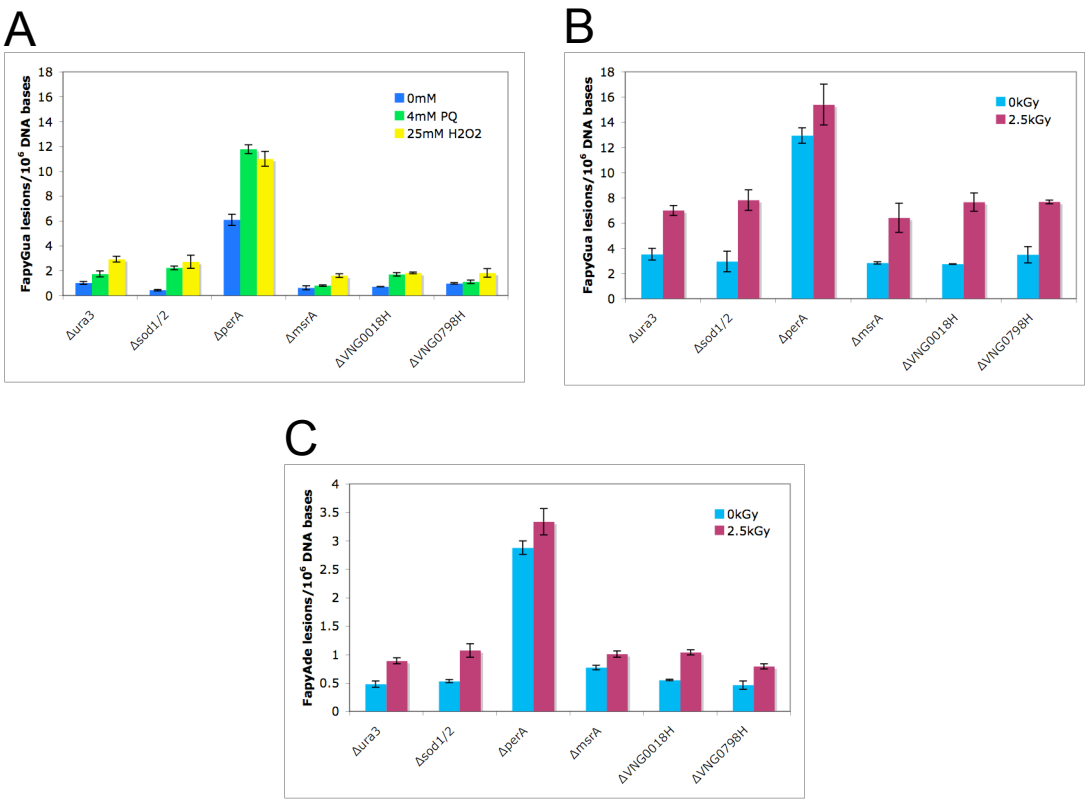


**Figure 3-5**. DNA lesions of *H. salinarum* NRC-1 mutants following oxidant treatment and IR. Oxidative DNA lesions were measured for strains Δ*ura3* (background strain), Δ*sod1/2*, Δ*perA*, Δ*msrA*, Δ*VNG0798H*, and Δ*VNG0018H* using GC/MS with isotope dilution. (A) FapyGua lesions were measured in DNA from cells treated with 4mM paraquat and 25mM hydrogen peroxide. (B) FapyGua lesions and (C) FapyAde lesions were measured in cells treated with 2.5kGy IR. These represent results from at least 3 independent cultures and uncertainty is presented as standard error.

lesions than the control strain during chemical oxidant treatment were Δ*sod1/2* and Δ*perA* (Figure 3-5 A). The Δ*sod1/2* mutant showed nearly a four-fold increase in FapyGua lesions after both superoxide and hydrogen peroxide treatments and Δ*perA* showed nearly a doubling in lesions with both treatments when compared to control cells. In contrast, we did not find significant differences in the number of FapyGua lesions in the control and mutant strains when cells were exposed to 2.5kGy of IR (Figure 3-5 B). FapyAde lesions were also measured for ROS mutants treated with IR, showing results similar to those of FapyGua (Figure 3-5 C). Untreated mutants showed the same level of DNA oxidative damage as the untreated controls with the exception of the Δ*perA* mutant, which accumulated a large number of oxidative bases even when grown under optimal conditions (Figure 3-5).

The level of protein damage in cells exposed to hydrogen peroxide, superoxide, and IR was determined in *H. salinarum* NRC-1 mutants and control strains (Figures 3-6 and 3-7) using the OxyELISA assay. We found quite a significant variation in the total amount of carbonyl residues from one experiment to the next. This seems to be a problem inherent to the assay itself since the same level of variation was also found when using the same cellular extract. To compare damage between sets of experiments we therefore considered the "fold" increases between challenged and untreated controls. The level of carbonyl residues in the proteins of mutants exposed to superoxide was not significantly different than that of the Δ*ura3* control strain (Figure 3-6 A). The only mutants with significant, yet modest, increases in carbonyl residues compared to the control strain were Δ*sod1/2* and Δ*perA*. This finding was

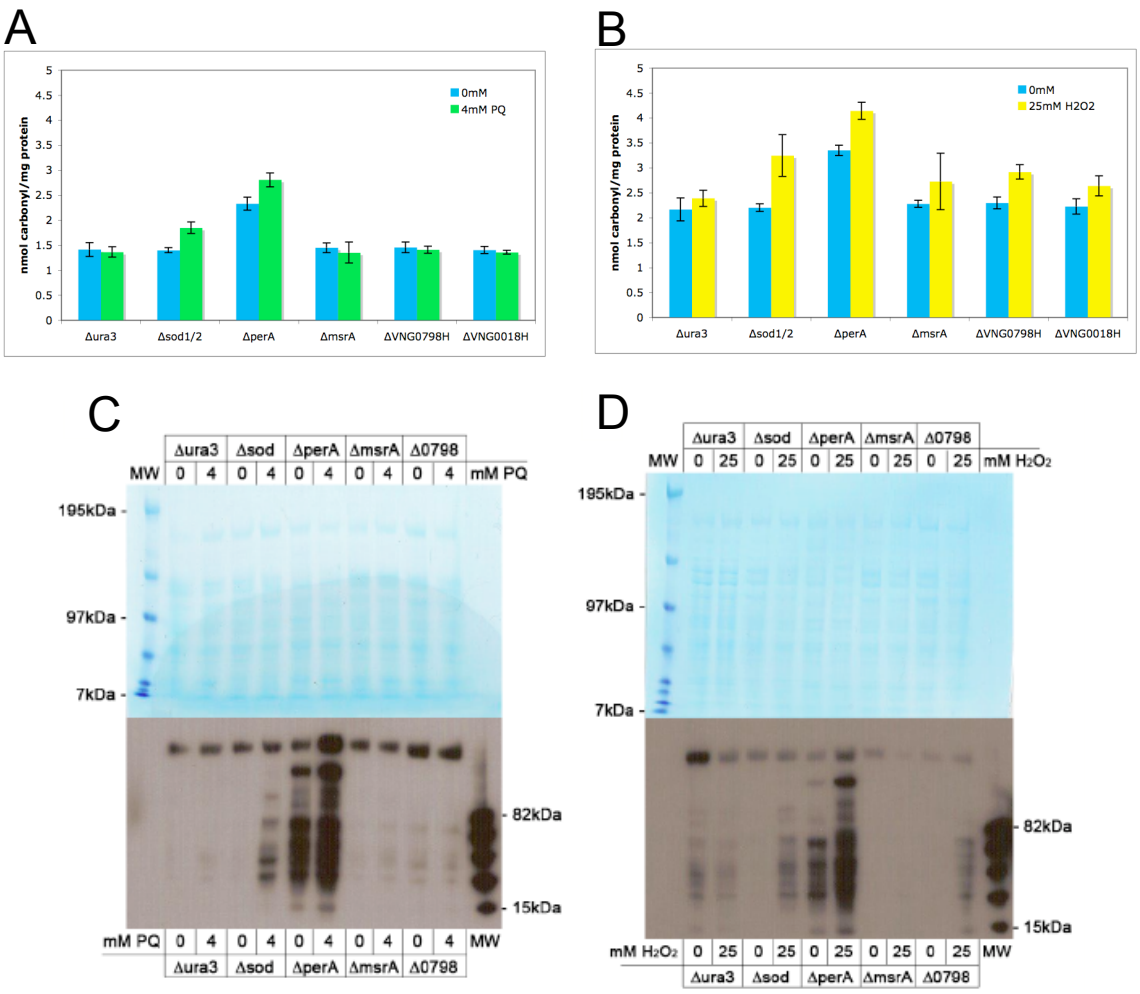


**Figure 3-6.** Protein oxidation of *H. salinarum* NRC-1 mutants following oxidant treatment. The level of protein carbonylation was determined in strains Δ*ura3* (background strain), Δ*sod1/2*, Δ*perA*, Δ*msrA*, Δ*VNG0798H*, and Δ*VNG0018H* with the OxyELISA or Oxyblot assays. OxyELISA assay with cells treated with (A) 4mM paraquat and (B) 25mM hydrogen peroxide. Oxyblot assay with cells treated with (C) 4mM paraquat and (D) 25mM hydrogen peroxide. OxyELISA results are the product of at least 3 independent cultures and uncertainty is presented as standard error.

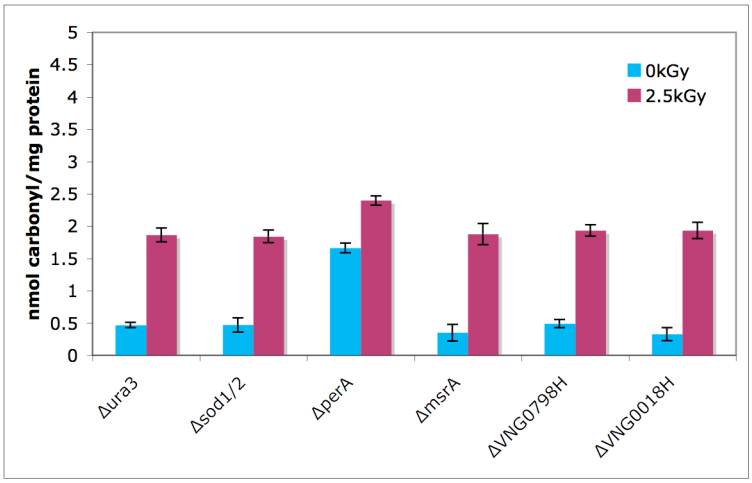


**Figure 3-7**. Protein oxidation of *H. salinarum* NRC-1 ROS scavenging mutants following 2.5kGy of IR. The level of protein carbonylation was measured in strains Δ*ura3* (background strain), Δ*sod1/2*, Δ*perA*, Δ*msrA*, Δ*VNG0798H*, and Δ*VNG0018H* with the OxyELISA assay. These results are the product of at least 3 independent cultures and uncertainty is presented as standard error.

confirmed by the Oxyblot assay, an immunodetection of protein carbonyl residues using western blotting (Figure 3-6 C). Treatment of mutant strains with hydrogen peroxide showed significant increases in carbonylation with three of the strains, Δ*sod1/2*, Δ*perA*, Δ*VNG0798H* (Figure 3-6 B and D), whereas there were not significant differences in the level of carbonyl residues between the control and mutant strains when exposed to IR (Figure 3-7).

To determine if inducible mechanisms were responsible for the resistance to IR observed in *H. salinarum* NRC-1, we pretreated Δ*ura3* cells with growth sub-inhibitory doses of hydrogen peroxide (10mM) and paraquat (2mM) before exposure to IR treatment [26]. Our analysis showed no difference in survival to 2.5 and 5kGy of IR, regardless of whether the cells were pretreated or not by either hydrogen peroxide or paraquat before irradiation (Figure 3-8).

*Discussion*

This work analyzed the roles of ROS scavenging enzymes and enzymes of the NER and BER pathways in preventing and repairing damage caused by oxidative stress. The survival of ROS deletion mutants to hydrogen peroxide and superoxide demonstrated the key roles of the peroxidases *perA* and *VNG0798H* in protection from hydrogen peroxide stress and superoxide dismutases *sod1* and *sod2* in protection from paraquat-induced superoxide stress [26]. Phenotypic characterization of the mutants was necessary for confirming the cellular function of these enzymes because our whole genome transcriptional analysis showed that most of the corresponding

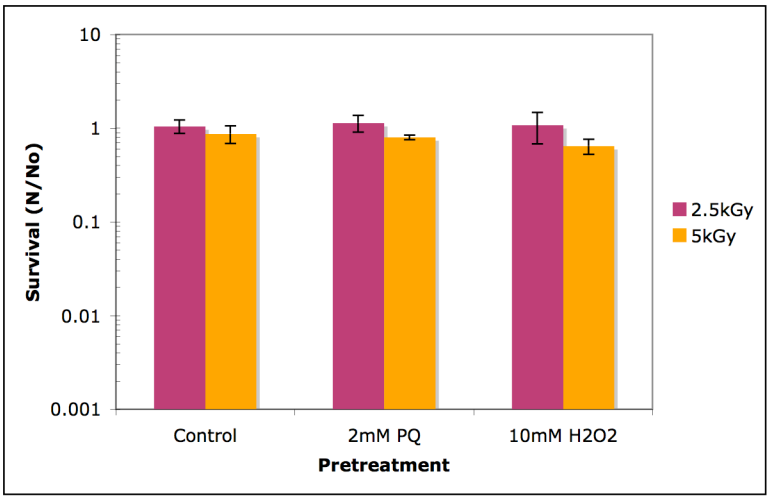


**Figure 3-8**. Survival of *H. salinarum* NRC-1 Δ*ura3* strain for IR following oxidant pretreatment. Cells were pretreated with 2mM paraquat or 10mM hydrogen peroxide (or untreated for the control) followed by exposure to 2.5 or 5kGy IR. Survival was determined by a plating assay. These represent results from at least 3 independent cultures and uncertainty is presented as standard error.

genes were upregulated under both oxidative stresses [26]. With paraquat exposure, *sod1* was upregulated in under 5 minutes while *sod2* took up to 80 minutes to be induced; under hydrogen peroxide stress, *perA* was highly upregulated [26].

A major finding of this work is that none of the ROS mutants we constructed were critical for survival to IR exposure, indicating that enzymatic ROS scavenging might not play a major role in resistance to IR in *H. salinarum* NRC-1. This is somewhat surprising given that it was reported that the survival of the extremely IR resistant *Deinococcus radiodurans* decreased after IR in deletion mutants lacking catalase or superoxide dismutase [86].

To identify DNA repair proteins key to the oxidative stress response of *H. salinarum* NRC-1, we analyzed the survival of NER and BER deletion mutants following treatment with chemical oxidants and IR. Multiple glycosylases in the BER pathway were knocked out, including double knockouts, to circumvent the multiplicity of those enzymes and the fact that some glycosylases might have overlapping roles in the detection of specific oxidative lesions. The UvrA, UvrB, and UvrC proteins, in contrast, functioned in the same NER pathway, so deletion of one gene abolishes the entire pathway, and therefore a single mutant from this pathway was tested [85]. None of the DNA repair mutants tested with up to 10mM paraquat and 30mM hydrogen peroxide showed a significant difference in survival when compared to the control strain, nor did the Δ*ogg* glycosylase and Δ*uvrA* mutants when treated with 2.5kGy of IR. These results seem to indicate that removal of oxidized bases might be

carried out by redundant DNA repair pathways in *H. salinarum* NRC-1, and therefore knocking out single, or tandem, DNA repair proteins was not enough to demonstrate a phenotype. The wide variety of DNA repair systems in *H. salinarum* NRC-1 makes it possible that if one enzyme/pathway is knocked out, another pathway for the repair of oxidized lesions can compensate and no decrease in survival is observed [10, 85].

The quantitation of FapyGua lesions in the DNA of *H. salinarum* NRC-1 mutant strains treated with oxidants or IR showed that oxidative stress resulted in DNA damage, but that the extent of this damage was not correlated with the survival of the mutants. Had this been the case, larger increases in damage following treatment should have been seen for the Δ*sod1/2*, Δ*VNG0018H*, and Δ*perA* mutant strains with paraquat exposure and the Δ*perA*, Δ*VNG0798H*, and Δ*VNG0018H* mutant strains with hydrogen peroxide exposure since those mutants showed significant decreases in survival following exposure to the corresponding chemical oxidants. Excluding the Δ*perA* mutant, there was no difference in the number of FapyGua or FapyAde lesions in *H. salinarum* NRC-1 DNA in cells treated with IR when the mutants and the background strains were compared. The high number of lesions found for Δ*perA*, including untreated cells, indicates an overall lack of fitness of the mutant even without exogenous oxidative stress. Oxidative stress from the cell's metabolic activity was enough to overwhelm the detoxification systems of the cell in the Δ*perA* mutant, demonstrating the constant need for hydrogen peroxide scavenging in *H. salinarum* NRC-1 cells.

Few studies have quantified individual DNA lesions after oxidative stress in prokaryotes. In *E. coli*, treatment with up to 1mM paraquat resulted in a drop in cell viability, yet there was no increase in the level of 8-OH-Gua, a DNA lesion commonly used as a marker for DNA oxidative damage [87]. When superoxide levels were increased in *E. coli* through limitation of superoxide dismutase activity, a 5x increase over normal superoxide levels resulted in DNA damage believed to be the result of increased free Fe in the cells [88]. This DNA damage was not directly measured, but inferred based upon the killing rate of cells exposed to superoxide [88]; deletion mutants lacking genes involved in DNA repair showed higher killing rates with hydrogen peroxide exposure than control cells, leading to the expectation that a decrease in survival was due to DNA damage [89]. Thus far, there has been little direct evidence of a correlation between survival and DNA damage as a result of chemical oxidation.

Our finding that there is no correlation between survival to oxidative stress and DNA damage is emphasized by the fact that the level of DNA damage is not significantly different between IR resistant and sensitive cells after IR exposure [59]. Roughly equivalent numbers of DNA strand breaks following a given dose of IR were found in cells regardless of their survival [59], indicating that DNA damage is dose-dependant and that survival is linked to the ability of the cells to repair DNA lesions. In *H. salinarum* NRC-1, IR-induced oxidative DNA lesions were repaired to pre-irradiation state within two hours [9]. We find here that despite similar levels of DNA damage,

there are major differences in the survival of *H. salinarum* NRC-1 ROS mutants following oxidant treatment.

In examining protein damage after oxidant treatment, we found an increase in protein carbonylation following oxidative stress but here again we did not find a correlation between the survival of the mutants and their corresponding protein oxidation levels. This demonstrates that although several of the ROS scavenging enzymes we tested are critical for survival to paraquat and hydrogen peroxide stress, they are not primarily involved in the protection of proteins from oxidative damage. This refutes the hypothesis that survival of ROS is mainly through protection of proteins from oxidation, at least for hydrogen peroxide and paraquat stress. Daly's group found that organisms that were resistant to IR showed greater protein protection during irradiation than IR sensitive organisms [7]. It was hypothesized that this protection may extend to other conditions that introduce oxidative stress, including desiccation and UV radiation [7], however we show here that this is not the case with chemical oxidant stress. In the case of hydrogen peroxide and paraquat, it is possible that the challenge caused by ROS in *H. salinarum* NRC-1 results in a more general damage to a variety of cellular pathways that, taken together, cause a strong challenge to the cells' survival. For example, the targeted oxidation of isopropylmalate isomerase, fumarase A and aconitase A, enzymes belonging to a family of labile [4Fe-4S] dehydratases, in *E. coli* strains impaired in hydrogen peroxide scavenging led to disruption of catabolic and biosynthetic pathways [90]. An alternate hypothesis might be that the observed cellular damage may be the result of the cellular

localization of the oxidative stress. With paraquat exposure, the drug interacts mainly with redox proteins near the membrane to produce superoxide at the perimeter of the cell [11]. Interaction with proteins related to cellular respiration can interfere with energy production, causing imbalance to the cell's redox homeostasis, or producing additional ROS molecules because of major electron transactions in this part of the cell. With hydrogen peroxide exposure, the oxidant must diffuse into the cell and interact with Fe to produce the majority of its damage to cellular targets [11]. Therefore, these oxidants may not be closely interacting with proteins while IR is able to deposit damage more evenly throughout the cell, thus damaging a wider array of proteins.

Paradoxically, exposure to IR resulted in a several folds increase in protein damage over the levels observed with hydrogen peroxide and paraquat stress in the control and mutant strains but no additional decrease in survival of those strains. The level of DNA oxidative lesions observed was similar for all the treatments. This result supports the idea that failure of the cellular subsystems might be the cause for cell death with paraquat and hydrogen peroxide before the level of protein oxidation, and therefore inactivation, throughout the cell becomes toxic.

Our novel finding that ROS scavenging enzymes are not required for IR survival suggests an alternate strategy for survival. To test the idea that the processes involved in cell detoxification from IR exposure might be inducible by oxidative stress, we examined if cell survival to IR increased when cells were pretreated with

growth sub-inhibitory doses of paraquat and hydrogen peroxide [26]. Whole-genome transcriptional analysis showed that at the sub-inhibitory dose of 0.25mM paraquat, genes for the *sod1* superoxide dismutase and the *VNG0798H* and *perA* peroxidases were upregulated, albeit at lower levels than with 4mM paraquat exposure (there was no equivalent exploration of sub-inhibitory hydrogen peroxide doses) [26]. The dose used in this study was 2mM paraquat, higher than the sub-inhibitory dose of Kaur et al [26], likely resulting in production of those enzymes. However, we did not observe increased survival of the pre-treated cells to IR, indicating that the response to oxidative stress from IR treatment is not inducible by oxidative stress. Previous work using low IR pretreatment followed by a high IR dose also failed to demonstrate an inducible response to IR stress [3].

Major findings from this work are two fold. First, we showed that there was no correlation of the level of DNA and protein oxidative damage with cell survival when *H. salinarum* NRC-1 cells were exposed to chemical oxidants. This is in contrast to findings with IR and the strong correlation between protein oxidative damage and cell survival previously established. This result suggests fundamental differences in the effective action of those stresses, possibly in the type of secondary ROS produced, and the localization of those ROS in cells. Second, we demonstrated that major ROS scavenging enzymes, critical for survival to hydrogen peroxide and paraquat stresses are not required for survival to IR stress. This is quite surprising and leads to the idea that cellular protection against IR might not be enzymatic in nature. Indeed, several recent works have advanced the idea of a critical role for Mn in the resistance to

radiation [7, 43, 59, 91]. Daly et al. [7] found that IR resistant microorganisms had a higher intracellular Mn/Fe ratio than radiation sensitive organisms, and we recently reported that *H. salinarum* NRC-1 intracellular Mn/Fe ratio was similar to that of *D. radiodurans* [34]. This aspect of resistance to IR is the subject of chapter 4.

# Chapter 4: Non-enzymatic scavenging of ROS is key for ionizing radiation resistance in *Halobacterium salinarum* NRC-1

*Introduction*

Ionizing radiation (IR) introduces damage to organisms both directly through ionizing reactions and indirectly through the radiolysis of water and subsequent production of reactive oxygen species (ROS) [13]. Radiolysis of water produces protons, free electrons, and hydroxyl radicals. Superoxide can be produced by those free electrons combining with $O_2$ [13]. A whole host of other ROS molecules can then be produced via secondary reactions (Figure 1-1, Chapter 1) [34]. Hydroxyl radicals react immediately with nearby molecules while less reactive ROS molecules are able to diffuse away from their site of production before causing damage [13].

Among the cellular targets of ROS damage are DNA, proteins, lipids, and carbohydrates. Hydroxyl radicals are the primary cause of damage to macromolecules in cells [12]. Hydrogen peroxide can be converted to hydroxyl radical via Fenton's reaction [12]. Since Fe, which also participates in the Fenton's reaction, is often associated with DNA and iron-sulfur clusters of proteins, hydroxyl radical production in those areas results in clustered damage to DNA and proteins [11]. Superoxide causes damage to cells primarily by attacking iron-sulfur clusters of

proteins, which increases the free iron in the cell and the production of hydroxyl radicals via Fenton's reaction [11]. In addition, cellular molecules that are damaged by ROS, such as protein peroxides, can propagate oxidative damage to other cell molecules [92].

The discovery of IR resistant organisms has led to intensive research into the basis of their resistance. Among the primary concerns are (1) what is the critical damage from IR that impacts cell survival and (2) how are resistant organisms able to limit or repair that damage? The strategies used by resistant organisms to protect themselves from IR are wide-ranging and not well understood [93]. The extremely radiation resistant bacterium *Deinococcus radiodurans* contains catalase and superoxide dismutase enzymes that seem to play a role in cell survival during IR exposure [86]. Survival after IR was found to decrease slightly in mutants lacking superoxide dismutase [86]. Early work on radiation resistance pointed to DNA protection and repair as being critical to survival to IR [94-96]. However, genome sequences and proteomics of IR resistant organisms did not reveal unique DNA repair systems as compared to IR sensitive organisms [31, 33, 97, 98]. Indeed, it was found that proteins necessary for repair of genome fragmentation in *D. radiodurans* following IR (RecA, RadA, PolI and PolIII) are homologous to those found in radiation-sensitive bacteria [99, 100].

Recent findings have demonstrated that DNA damage from IR, in the form of double strand breaks, is introduced at the same levels in both radiation sensitive and radiation

resistant organisms [35, 101]. In contrast, differences have been found between radiation sensitive and radiation resistant bacteria in the levels of protein damage following IR. Survival during IR exposure correlates well with low protein oxidation [6, 7, 59]. In mouse cell lines exposed to hydroxyl radicals produced by IR, proteins were damaged before DNA and lipids [36]. This indicates that the most relevant IR targets in cells regarding survival might be proteins, and that radiation resistant organisms are better able to protect their proteins than sensitive organisms. Protected enzymes involved in repair of DNA and other macromolecules damaged by IR can then start the repair processes required for cell survival [34].

Another significant finding is that high Mn/Fe ratios in prokaryotes correlate with radiation resistance [34]. There is evidence *in vitro* that Mn(II) complexes can limit ROS damage through the disproportionation of hydrogen peroxide to $O_2$ and $H_2O$ [42] and that manganous phosphate has superoxide scavenging activities [43]. Additionally, Mn supplementation can rescue growth and other defects of bacterial cells lacking superoxide dismutase [39, 102]. The protection offered by Mn has been hypothesized to be through out-competing Fe in binding to active sites of proteins and therefore limiting Fenton's reactions that can occur in close contact with proteins [11]. An alternative hypothesis was proposed for the protective effect of Mn through the redox cycling [7]. Mn(II) can be oxidized by superoxide to produce hydrogen peroxide and Mn(III), and this Mn(III) can be reduced back to Mn(II) through the break down of hydrogen peroxide to oxygen and $H^+$. This Mn cycling could result in the production of less damaging ROS molecules than hydroxyl radical that is

produced by Fe cycling [7]. Halides in halophilic radiation resistant organisms have also demonstrated protection from IR for both DNA and proteins [9], thus establishing the relevance of other small molecules in radiation protection. An additional piece of evidence for chemical scavenging of ROS is that enzyme-free cell lysates of *D. radiodurans* have been shown to protect more sensitive organisms from IR [103]. This raises the question of what non-enzymatic molecules are responsible for cellular protection from IR.

In this study, we used as a model system the halophilic archaeon *H. salinarum* NRC-1. It grows optimally in 4M NaCl and accumulates intracellular salts to the same concentration as its environment [1]. This organism is resistant to IR with a $D_{10}$ (dose of ionizing radiation corresponding to 10% survival) of 5kGy [9]. Among attributes that contribute to the IR resistance of *H. salinarum* NRC-1 are high levels of membrane pigments, particularly bacteriorubrin that have the ability to scavenge ROS [3]. *H. salinarum* NRC-1 also demonstrates efficient repair of DNA double strand breaks following IR exposure [3, 9]. This is due in part to the multiple copies of its genome [51], providing a large amount of templates for the repair of double strand breaks by homologous recombination. Our findings that ROS scavenging enzymes such as catalase, peroxidase, and superoxide dismutase are not critical for *H. salinarum* NRC-1's survival of IR indicate that some other protection system is operating (see Chapter 3). Intracellular salts have been found to offer protection to *H. salinarum* NRC-1 from IR, most likely due to interactions between halides and hydroxyl radical to produce much less reactive halide radicals [9]. This provides

further evidence that non-enzymatic systems for preventing IR damage may also be present in *H. salinarum* NRC-1.

The radiation sensitive organisms used in this study are the bacteria *Escherichia coli* and *Pseudomonas putida*. *E. coli* is a gram-negative bacterium with an ionizing radiation $D_{10}$ dose of 0.7kGy [59]. *P. putida* is also a gram-negative bacterium with a $D_{10}$ of 0.25kGy [59]. Neither organism is halophilic, so they contain much lower levels of intracellular salt than *H. salinarum* NRC-1; indeed, at salt concentrations as low as 0.4 M NaCl, *E. coli* experiences salt stress [104]. The Mn/Fe concentration ratio for *E. coli* is 0.0072 and for *P. putida* it is 0.0001 [59]. *H. salinarum* NRC-1's Mn/Fe ratio is 0.27, and is similar to *D. radiodurans* high ratio of 0.24 [9]. These ratios all follow the trend of low Mn/Fe ratios for radiation sensitive organisms and higher ratios for radiation resistant organisms [59].

This research focused on elucidating the role small molecules and chemical scavengers play in preventing IR damage in the radiation resistant *H. salinarum* NRC-1. The enzyme-free cell extracts of *H. salinarum* NRC-1 was assayed for radioprotection activities of DNA integrity and protein activity after IR treatment and compared with those of *E. coli* and *P. putida*. Analysis of *H. salinarum* NRC-1 enzyme-free cell extracts with inductively coupled plasma mass spectrometry (ICP-MS) and ion chromatography revealed high levels of Mn and phosphates indicating a significant potential for ROS scavenging in *H. salinarum* NRC-1. High levels of specific nucleosides were found by LC-MS analysis in *H. salinarum* NRC-1 enzyme-

free cell extract that were not reproduced in similar extracts from *E. coli* or *D. radiodurans*. These studies contributed novel findings on the molecular mechanisms underlying the radiation resistance of *H. salinarum* NRC-1 and provided exciting new directions for future investigations.

## *Materials and Methods*

### *Preparation of enzyme-free ultrafiltrates*

Enzyme-free ultrafiltrates (UF) were prepared for *H. salinarum* NRC-1, *E. coli*, and *P. putida* grown in GN101 (250g/L NaCl, 20g/L $MgSO_4 \bullet 7H_2O$, 2g/L KCl, 3g/L Na citrate, 10g/L Oxoid brand peptone), pH 7.2 with the addition of 1ml/L trace elements solution (31.5mg/L $FeSO_4 \bullet 7H_2O$, 4.4mg/L $ZnSO_4 \bullet 7H_2O$, 3.3mg/L $MnSO_4 \bullet 7H_2O$, 0.1mg/L $CuSO_4 \bullet 5H_2O$), LB (10g/L Tryptone, 5g/L Yeast extract, 10g/L NaCl, pH 7.0), and TGY (10g/L Bacto-tryptone, 5g/L Yeast extract, 1g/L glucose, pH 7.0) mediums, respectively. For each cell type, 15.5g of wet weight cells were resuspended in 35ml ddH2O and passed through a French press at 900 psi to lyse cells. Cell lysates were centrifuged at 12,000 x g at $4^oC$ for 60 minutes, the supernatant was recovered, and protein concentration of the three UFs was determined to be approximately 17mg/ml using the BioRad Bradford Assay (Hercules, CA). An ultracentrifugation was then performed on 10ml aliquots of each cell lysate at 50,000 rpm, $4^oC$ for 48 hours. The supernatant was recovered and spun through 3kDa filter tubes (Millipore, Billerca MA) at 4000 x g, $4^oC$, 45 minutes to remove macromolecules over 3kDa in size. Cell lysates were then boiled for 30 minutes to ensure the loss of all enzymatic activity. Ultrafiltrates were concentrated

in a speed vacuum until they reached 5x concentration. Samples were aliquoted and stored at -20$^{o}$C. The 1x (or 100%) UF concentration was calculated from the ratio of the measured protein concentration in the extract before ultracentrifugation and an estimate of protein concentration in the wet cell mass using total cell numbers and 155 fg protein per cell [105]. Further dilutions/concentrations of the extract as the result of ultracentrifugation and ultrafiltration steps were taken into account in the calculation. We found that the UF was approximately 1.2 fold more dilute than the intracellular milieu. *D. radiodurans* UF was provided by Dr. Daly (Uniformed Services University of Health Sciences) at 4x concentration.

*pUC19 DNA protection assay*

The ability of *H. salinarum* NRC-1, *E.coli* and *P. putida* UFs, as well as KCl and KBr buffers, to protect pUC19 DNA (New England Biolabs, Ipswich, MA) from strand breakage after IR was determined as follows. pUC19 DNA was irradiated at a final concentration of 40ng/µl in the UF and KCl/KBr salt solutions. The pUC19 DNA was added to UFs that were at either 100% or 20% strength relative to their concentration following ultracentrifugation. The pUC19 DNA was added to salt buffers at final concentrations of 4, 3.8, 1.6, 0.8, and 0.4 M KCl and KBr. DNA in 25mM phosphate buffer, pH 7.0 served as control. These *in vitro* solutions were irradiated using a $^{60}$Co gamma source (Uniformed Services University of the Health Sciences, Bethesda, MD) and samples were taken at 0, 0.1, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, and 15kGy doses. The resulting DNA fragments were electrophoresed on a 0.9% agarose TBE gel and visualized using ethidium bromide staining.

*Enzyme activity protection assay*

The ability of *H. salinarum* NRC-1, *E. coli*, and *P. putida* UFs and KCl and KBr buffers to protect the activity of restriction enzyme *DdeI* (New England Biolabs, Ipswich, MA) from damage due to IR was assessed as follows. Buffer solutions were tested with *DdeI* at a final concentration of 1.5 U/µl. *DdeI* was added to the UFs that were at 20% of the strength relative to their concentration following ultracentrifugation. *DdeI* was irradiated in salt buffers at final concentrations of 0.8 and 0.4 M KCl and KBr. The irradiations were performed using a $^{60}$Co gamma source (Uniformed Services University of the Health Sciences, Bethesda, MD) and samples were taken at 0, 0.25, 0.5, 1, 2, 4, 6, 8, 10, 12, and 15 kGy doses. Samples were kept on ice until digestion of 1 µg of pUC19 DNA using 2.5 U of enzyme from each IR dose at 37$^{o}$C for 1 hour. The resulting pUC19 DNA fragments were electrophoresed on 1% agarose TBE gels and visualized with ethidium bromide staining.

*Determining composition of UF's*

**Free amino acid and total amino acid concentration**

Free amino acid and total amino acid concentrations in the UF of *H. salinarum* NRC-1, *E.coli*, and *P. putida* UFs were determined using the ninhydrin assay [106]. Briefly, tryptophan standard solutions were made ranging from 0 to 200nmol tryptophan and the ultrafiltrates were diluted 1:100 in dd$H_2O$ for the determination of free amino acid concentration. Ninhydrin reagent (Sigma-Aldrich, St. Louis, MO) was added to each solution and boiled for 20 minutes. Isopropynol was added to 50%

final concentration and the absorbance of each sample was read at 570 nm. A standard curve was constructed based on the tryptophan standards to determine free amino acid concentration in the UFs. For the determination of total amino acid concentration, an acid hydrolysis as described in [107] was carried out before assaying free amine concentration with the ninhydrin assay. In short, the UFs were diluted 1:10 in dd$H_2O$ and an equal amount of 10.5N HCl was added. The mixture was flushed with nitrogen, sealed in a glass ampoule, and incubated at 110$^oC$ for 24 hours. The sample was then diluted 1:10 with dd$H_2O$, ninhydrin reagent (Sigma-Aldrich, St. Louis, MO) was added to each solution and amino acid concentrations were measured as described above. The analysis of amino acid concentrations of the UFs was performed by Kimberly Webb.

**Nucleoside and nucleotide composition**

Nucleoside and nucleotide composition of *H. salinarum* NRC-1, *E.coli*, and *D. radiodurans* UF were determined by LC-MS in collaboration with Dr. Allen Place at the Center of Marine Biotechnology, UMBI. Twenty microliters of ultrafiltrates were injected onto an Agilent Prep C18 column (LiChrosphere 125 mm x 4mm, 5 mm bead size RP-18, Agilent; Santa Clara, CA) at 45°C and subjected to a 0.9 mL / min. isocratic elution with 0.1 M Triethanolamine acetate pH 6.5 using an Agilent 1100 HPLC (Agilent 1100 LC/MS system; Agilent, Santa Clara, CA). UV peaks were detected based on their UV absorbance at 254 and 270 nm. For the MS analysis, the flow from the HPLC (0.9mL / min) was pumped into the MS electrospray chamber with the addition of 0.1 mL / min. of 1% formic acid in methanol. The MS was set up for optimal nucleotide/nucleoside ionization by using a fragmentor voltage of 350 V and a capillary voltage of 4000V. At these settings doubly charged ions were minimized and the total

ion abundance of the singly charged parent was at a maximum. Nucleosides standards (adenosine, cytidine, deoxyadenosine, deoxycytidine, deoxyguanidine, deoxythymidine, guanidine, and uridine) purchased from Sigma (Sigma; St Louis, MO) were run under the same condition for each ultrafiltrate analysis.

**Chemical composition**

Mn, Fe, and $PO_4$ concentration in *H. salinarum* NRC-1, *E.coli*, and *P. putida* UF were determined using ICP-MS (Mn, Fe) and Ion chromatography ($PO_4$) at the Division of Environmental Health Engineering, JHU School of Public Health. For ICP-MS analysis, 50µl of UF was transferred to a pre-cleaned 15ml polystyrene tube and diluted to a final volume of 1.5ml with 1% $HNO_3$ + 0.5% 1N HCl. Internal standards (Mn or Fe) were added to each sample to monitor for sample matrix effects of the plasma. Analysis was performed with an Agilent 7500ce Induced Coupled Plasma-Mass Spectrometer (Agilent Technologies; Santa Rosa, CA). A standard calibration curve was generated from multi-element standard (Elements INC; Shasta Lake, CA) at the following concentrations: 0, 1, 5, 10, 50, 100, 500, 1000 µg/L. Reported sample concentrations of Mn and Fe were blank and dilution corrected. SRM 1643e (NIST; Gaithersburg, MD) was used to test the accuracy of sample preparation, and was prepared in the same manner as the samples.

For ion chromatography analysis, 25µl of UF was transferred into a pre-cleaned Dionex IC vial (Dionex Corp; Sunnyvale, CA), MilliQ water was added up to 1.5mL final volume, and the sample was vortexed to ensure thorough mixing. Analysis was performed using a Dionex DX600 Ion Chromatograph (Dionex Corp; Sunnyvale,

CA). A standard calibration curve was generated from a multi-anion solution (Elements INC; Shasta Lake, CA) containing the anion of interest ($PO_4$). Concentrations of the calibration curve were as follows: 0, 1, 2, 4, 6, 12, 16, 20 µg/ml. Samples were run on an IonPac AS14A Anion exchange column (4 x 250mm) (Dionex Corp; Sunnyvale, CA) and AS14A Guard (3 x 150mm) (Dionex Corp; Sunnyvale, CA) column using 1.08mM $Na_2CO_3$ and 1.02mM $NaHCO_3$ as eluent. Samples were suppressed using an ASRS 4mm suppressor (Dionex Corp; Sunnyvale, CA) with a current of 100mA. Samples were eluted for 30 minutes to ensure complete anion exchange. Anion retention times (+/-5%) were determined based upon the certificate of analysis for the column. Sample concentrations of $PO_4$ were reported as the average of the two replicates after blank and dilution correction.

*Results*

Enzyme-free ultrafiltrates (UF) were prepared for the radiation resistant archaeon *H. salinarum* NRC-1 and the radiation sensitive bacteria *E. coli* and *P. putida*. The UFs represented the <3kDa fraction of the cells; the 100% UF represented a 1.2 fold dilution of the intracellular milieu and 20% UF represented a 6 fold dilution. The ability of these UFs to protect macromolecules against IR damage was first tested with DNA. Plasmid pUC19 DNA was added to 100% UFs from the three organisms and irradiated from 0 to 15kGy of IR (Figure 4-1 A). Agarose gel electrophoresis of plasmid DNA after IR revealed the accumulation of DNA strand breaks seen through the progression from the superocoiled form of the plasmid to the open-circle form (representing one strand break), the linear form (representing two strand breaks), and

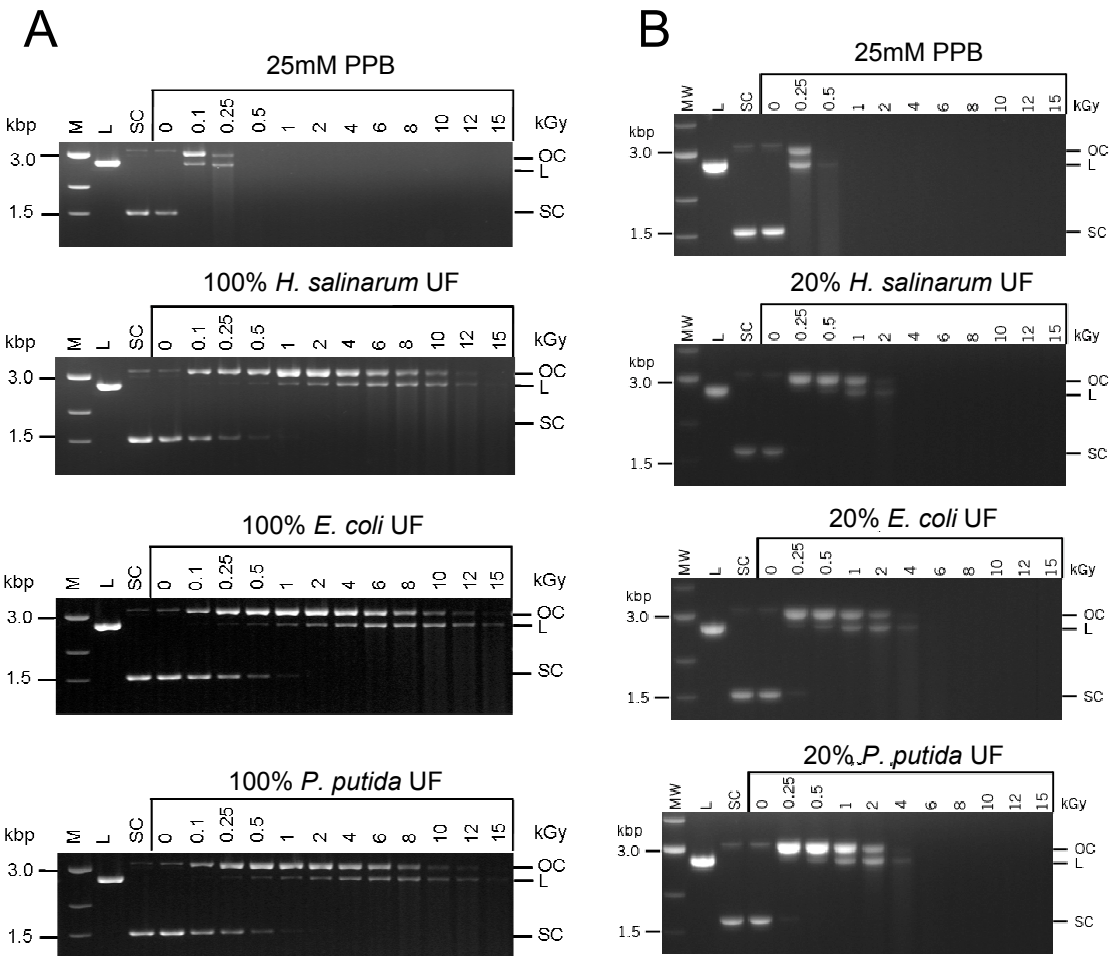


**Figure 4-1.** Agarose gel electrophoresis illustrating DNA protection from IR by enzyme-free UFs of *H. salinarum* NRC-1, *E. coli*, and *P. putida*. The plasmid pUC19 was irradiated in 100% UFs (A) and 20% UFs (B) up to 15 kGy. The accumulation of damage to the DNA can be seen through its progression from supercoiled to open-circle to linear forms. PPB = phosphate buffer; UF = utrafiltrate; M = molecular weight marker; L = linear; SC = supercoiled; OC = open circular plasmid.

the eventual degradation of the DNA with the absence of bands on the agarose gel. In the control, constituted of plasmid DNA in 25mM phosphate buffer, the last DNA band was visible for 0.25kGy of irradiation. In contrast, the three 100% UFs protected the DNA from complete degradation to 15kGy of IR, with supercoiled and linear DNA bands still visible for 12 and 15kGy.

To tease out differences in their DNA protection capabilities, we assayed diluted UFs at a final concentration of 20% (Figure 4-1 B). Our results showed protection against DNA degradation up to 4kGy with the *E. coli* and *P. putida* UFs while *H. salinarum* NRC-1 UF prevented DNA degradation to 2 kGy. This illustrated that all three UFs offered similar levels of protection to DNA after IR exposure.

In previous work, the importance of salts on radiation protection had been shown by measuring DNA oxidative lesions after IR *in vivo* in *H. salinarum* NRC-1 [9]. Here, we irradiated pUC19 DNA in KCl (Figure 4-2 A) and KBr (Figure 4-2 B) buffers ranging in concentration from 0.4 to 4 M. Our results showed low level protection against DNA strand break and degradation by KCl, with protection increasing with salt concentration. A concentration of 0.4M KCl prevented DNA degradation to about 0.25kGy, and to 1kGy at a concentration of 4M KCl. KBr, in contrast, afforded better DNA protection, up to 6 or 8 kGy, and it was not concentration dependent.

To test the hypothesis that protein protection is key to ionizing radiation resistance [7], we examined the ability of our UFs to protect enzyme activity during IR. The

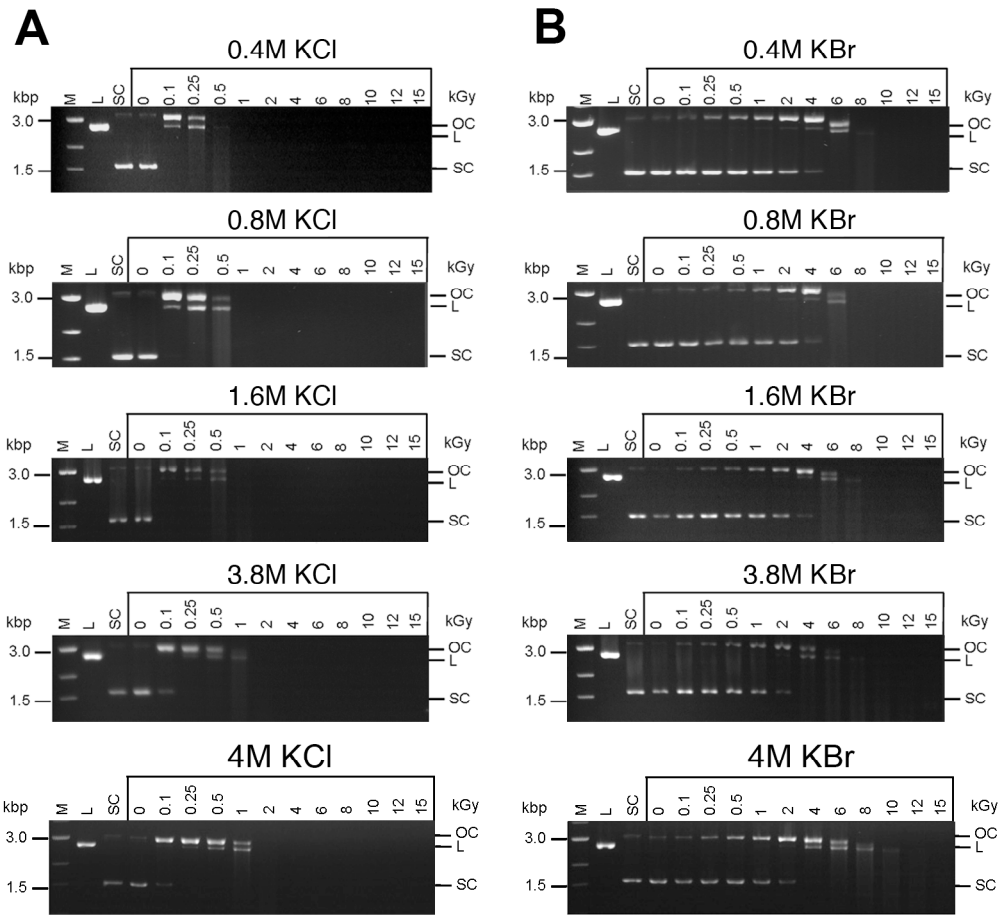


**Figure 4-2.** Agarose gel electrophoresis illustrating DNA protection from IR by increasing concentrations of salt buffers. pUC19 DNA was irradiated in 0.4, 0.8, 1.6, 3.8, and 4 M KCl (A) and KBr (B) buffers up to 15 kGy. The accumulation of DNA strand breaks with irradiation resulted in the progression from supercoiled to open-circle to linear forms of the plasmid. M = molecular weight marker; L = linear; SC = supercoiled; OC = open circular plasmid.

restriction enzyme *DdeI* was irradiated in 20% *H. salinarum* NRC-1, *E. coli*, and *P. putida* UFs up to 15kGy of gamma ray. Samples of the enzyme mixture were taken at each dose and subsequently used to digest pUC19 DNA; the resulting DNA fragments were visualized by agarose gel electrophoresis (Figure 4-3). Enzyme samples that retained activity showed banding patterns similar to the non-irradiated enzyme control. The *H. salinarum* NRC-1 UF afforded great protection to the restriction activity of *DdeI*, maintaining nearly full activity up to 10kGy. The *E. coli* and *P. putida* UFs, in contrast, provided full restriction activity to only the 0.5kGy dose.

The ability of KCl and KBr salt buffers to protect enzyme activity during IR was also examined. When the 0.8M KCl buffer was tested for its ability to protect restriction enzyme activity, it was found to provide protection up to the 1 or 2kGy dose of IR. The 0.8M KCl represent the salt concentration in the *H. salinarum* NRC-1 20% UF used in the enzyme assay described above, demonstrating that the protection from the *H. salinarum* NRC-1 UF is not merely due to its high salt content. The 0.8 M KBr buffer showed no protection for any irradiated samples. This is in contrast to the DNA protection assay that showed more protection of pUC19 DNA when incubated with KBr rather than KCl.

We next analyzed the composition of the UFs from *H. salinarum* NRC-1, *E. coli*, *P. putida* and *D. radiodurans*. Concentrations of free and total amino acids in the UFs were quantified using the ninhydrin assay before and after acid hydrolysis (Figure

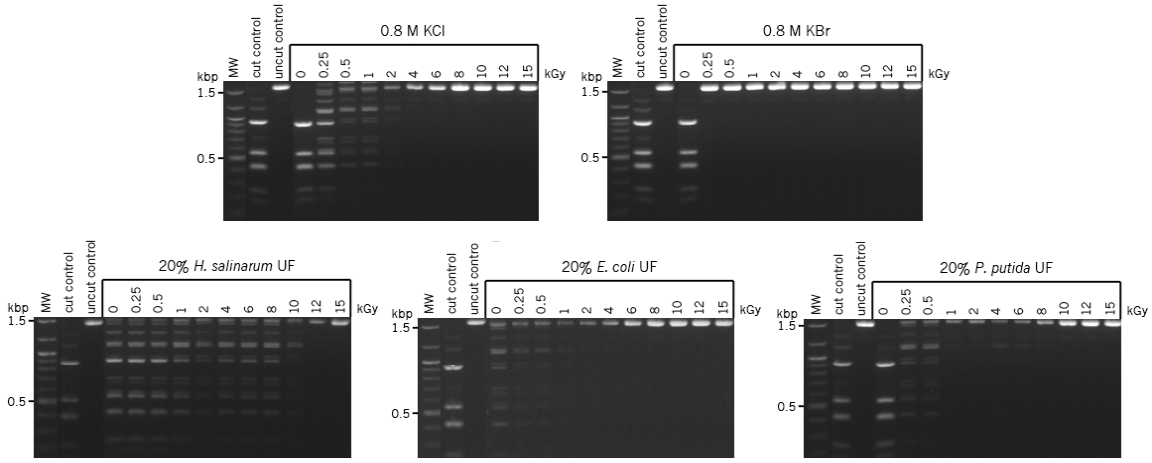


**Figure 4-3.** Agarose gel electrophoresis representing the residual restriction enzyme activity following IR exposure in salt buffers or 20% enzyme-free UFs from *H. salinarum* NRC-1, *E. coli*, or *P. putida*. The restriction enzyme *Dde I* was irradiated up to 15kGy in 0.8M KCl and KBr salt buffers and in 20% UFs of *H. salinarum* NRC-1, *E. coli*, and *P. putida*. Samples of the enzyme at each dose were used to digest pUC19 plasmid DNA and the fragments were analyzed by agarose gel electrophoresis. MW = molecular weight; UF = ultrafiltrate.

4-4). Free amino acids were most numerous in *D. radiodurans* 100% UF at 6.0 μM followed by *H. salinarum* NRC-1, *P. putida*, and *E. coli* 100% UFs at 3.2, 2.1, and 1.3 μM, respectively. After acid hydrolysis, the levels of total amino acids in *H. salinarum* NRC-1, *E. coli*, and *P. putida* UFs was less than 1μM greater than the free amino acid concentrations, and this difference corresponds to the amount of amino acids that were formerly combined in peptides. In contrast, *D. radiodurans* UF showed an increase of 9μM amino acids after acid hydrolysis, indicating that these amino acids were likely combined in short peptides.

Liquid chromatography and mass spectrometry was used to identify differences in composition and concentration of nucleosides in the UF of *H. salinarum* NRC-1, *E. coli*, and *D. radiodurans* (Figure 4-5). The spectra obtained for *E. coli* UF is complex with no major peaks whereas the spectra of *H. salinarum* NRC-1 and *D. radiodurans* UF showed several high abundance peaks. The elution times of *H. salinarum* NRC-1's three major peaks were consistent with those of pyrimidines, possibly uracil/uridine or cytosine/cytidine (Figure 4-5 A). *D. radiodurans* UF had three peaks also in the pyrimidine range and two peaks that may correspond to adenosine or deoxyadenosine. The integrated areas of the peaks give an approximation of their relative abundances, and the three most abundant peaks for each organism were compared (Figure 4-6). The peaks in *H. salinarum* NRC-1 UF were between three and six times larger than the largest peak in *E. coli* UF, and *D. radiodurans* UF contained peaks that were up to eight times the area of *E. coli* UF's largest peak. This

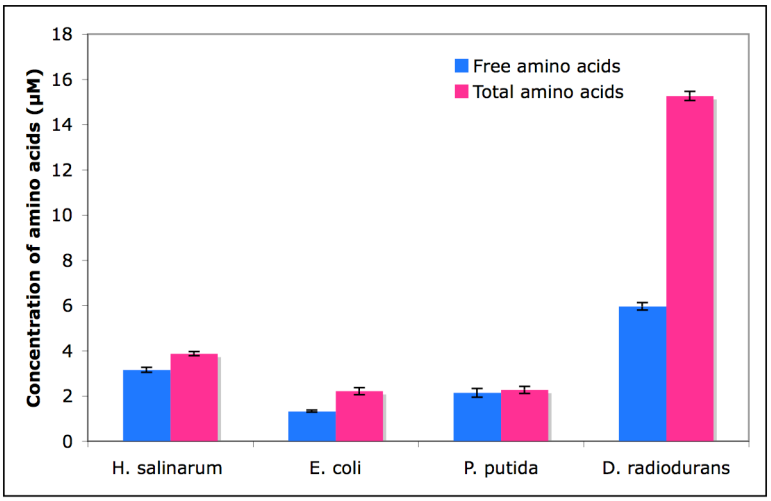


**Figure 4-4**. Concentration of free and total amino acids in *H. salinarum* NRC-1, *E. coli*, *P. putida*, and *D. radiodurans* 100% UF. The concentration of free amino acids in the UF was measured through the ninhydrin assay while total amino acid concentration was determined by an acid hydrolysis of peptides followed by the ninhydrin assay. Results are the average of at least 3 independent replicates and uncertainties are presented as standard deviation.

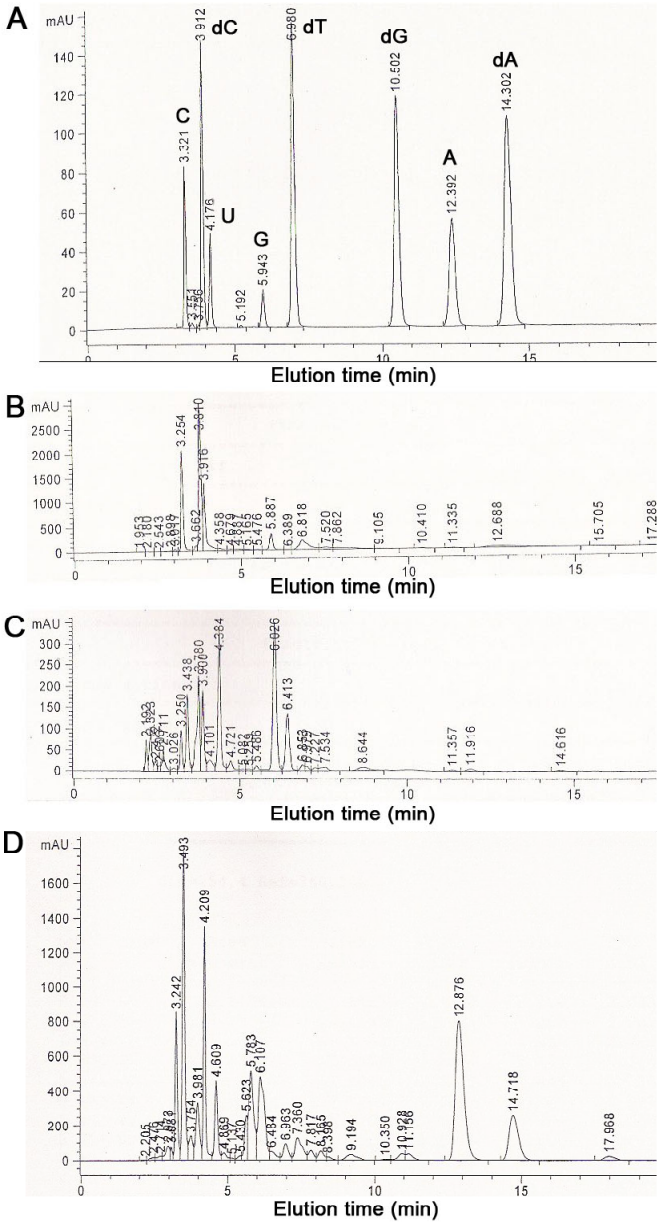


**Figure 4-5**. UV spectra at 254nm of liquid chromatography elution profile analyzed using isocratic separation for (A) DNA and RNA nucleoside standards (B) *H. salinarum* NRC-1 UF (C) *E. coli* UF and (D) *D. radiodurans* UF. The identities of nucleoside standard peaks in (A) are noted next to the peaks. mAU = milliAbsorbance units; C = cytidine; dC = deoxycytidine; U = uridine; G = guanidine; dT = deoxythymidine; dG = deoxyguanidine; A = adenosine; dA = deoxyadenosine.

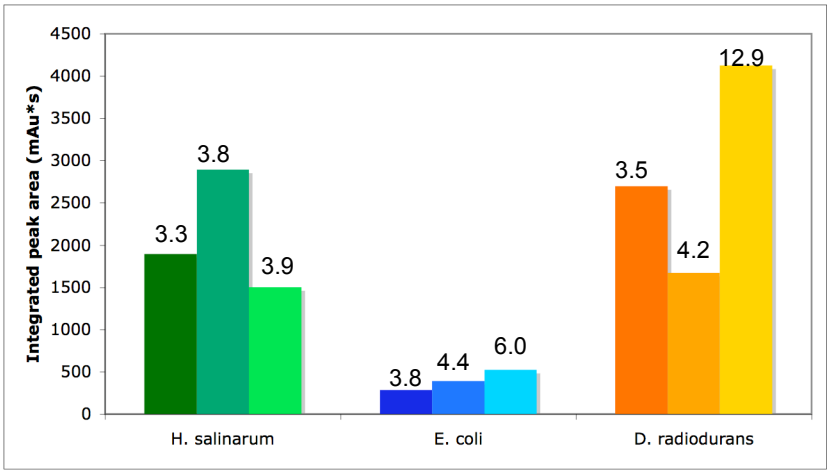


**Figure 4-6**. Amount of the three most abundant nucleosides in *H. salinarum* NRC-1, *E. coli*, and *D. radiodurans* 100% UF. Based upon the UV spectra at 254nm of the liquid chromatography profile of UF separated isocratically, the integrated areas of the three largest peaks are presented from *H. salinarum* NRC-1, *E. coli*, and *D. radiodurans*. Data labels are the elution times in minutes for each peak. mAU = milliabsorbance units; s = seconds (of elution time).

indicates that these specific nucleosides accumulate at much higher levels in the UF of radiation resistant organisms.

The level of Mn and Fe in the UFs was analyzed using inductively coupled plasma mass spectroscopy (ICP-MS) and the amount of $PO_4$ in the UFs was determined through ion-exchange chromatography (Table 4-1). Mn levels were 100x more concentrated in *H. salinarum* NRC-1 UF than *E. coli* and *P. putida* UF, while the Fe levels of *H. salinarum* NRC-1 UF were 5 to 10 times as concentrated. Despite *H. salinarum* NRC-1 UF containing more Fe than the other two UFs, its Mn/Fe ratio was still higher at 5.76 while *E. coli* and *P. putida* UF Mn/Fe ratios were 0.48 and 0.26, respectively. The concentration of $PO_4$ was only 3.47mM in the *H. salinarum* NRC-1 UF and around 5 times more concentrated in *E. coli* and *P. putida* UF at 15.29 and 18.92mM, respectively.

*Discussion*

This focus of this work was a better understanding of the functions and identity of non-enzymatic IR defenses used by radiation resistant organisms. Our findings that none of the ROS scavenging enzymes, including catalase, peroxidase, and superoxide dismutase, were essential for *H. salinarum* NRC-1's survival after IR exposure points to a critical role for non-enzymatic mechanisms of ROS scavenging in IR resistance for this organism (see Chapter 3).

**Table 4-1**. The concentration of Mn, Fe, and $PO_4$ in *H. salinarum* NRC-1, *E. coli*, and *P. putida* in 100% UF. Mn and Fe concentrations were determined by ICP-MS and $PO_4$ concentration was determined by ion chromatography.

| 100% UF | µM Mn | µM Fe | mM $PO_4$ |
|---|---|---|---|
| *H. salinarum* NRC-1 | 13.70 | 2.38 | 3.47 |
| *E. coli* | 0.11 | 0.23 | 15.29 |
| *P. putida* | 0.14 | 0.54 | 18.92 |

Here we report that the non-enzymatic, <3kDa, cellular extract from *H. salinarum* NRC-1, *E. coli*, and *P. putida* provided the same level of protection to plasmid DNA exposed to high doses of IR. This is in contrast to the level of radiation resistance reported for those organisms. The $D_{10}$ of *E. coli* is 0.7kGy and the $D_{10}$ dose of *P. putida* is 0.25kGy [59], while the $D_{10}$ of *H. salinarum* NRC-1 is 5kGy [9]. This finding is in agreement with work demonstrating that the amount of DNA fragmentation is not different between radiation sensitive and radiation resistant organisms when exposed to the same dose of IR [59, 101]. The linear density of double strand breaks of DNA per IR dose and per Mbp is the same regardless of IR sensitivity of the organism [35]. In addition, the amount of DNA damage imparted by IR is dose dependant [9, 101], indicating that DNA of IR resistant organisms is not more impervious to damage, but rather more efficiently repaired than IR sensitive organisms, resulting in higher survival.

Our previous work has demonstrated that salt accumulation in *H. salinarum* NRC-1 protects DNA from oxidative lesions more than *D. radiodurans*, however it is still less resistant to IR than *D. radiodurans* [9]. Our finding here that KBr is better able than KCl to protect against DNA fragmentation from IR is likely due to detoxification of hydroxyl radicals by electron transfer to form halide radicals [9]. These halide radicals are much less reactive than hydroxyl radical, thus limiting the damage that is introduced to other cellular macromolecules, and bromide provides better protection to DNA because hydroxyl radicals react more quickly with bromide than chloride [9]. While Br was unable to offer any protection to proteins during IR, Cl offered

moderate protection to enzyme function, yet not as much as the *H. salinarum* NRC-1 UF. Our finding that neither Cl nor Br could account for the high level of proteins protection in *H. salinarum* NRC-1 during IR exposure indicates that this ROS scavenging by halides is likely not essential for *H. salinarum* NRC-1 survival of IR.

Recent models have proposed that protein oxidation may be the primary challenge to which radiation resistant organisms are well adapted [7, 34]. In this study, we showed that *H. salinarum* NRC-1 UF protected protein activity to very high doses of IR, up to 12kGy. In contrast, UFs from *E. coli* and *P. putida* protected the restriction enzyme activity only to 0.5kGy. This lends support to the hypothesis that protein protection is key to survival of IR [7]. This also explains why repair of DNA is possible in IR resistant organisms but not IR sensitive organisms in spite of them containing the same repair pathways: repair proteins are protected from IR damage and therefore are still functional in radiation resistant organisms.

Another compound possibly implicated in protein protection in *H. salinarum* NRC-1's UF is Mn. Indeed, we found that Mn in *H. salinarum* NRC-1 UF was 100 times more concentrated than in those of *E. coli* and *P. putida* UF. *In vitro* studies have reported ROS scavenging for $MnPO_4$ and Mn complexes [38], and we therefore suggest that Mn plays a major role in ROS scavenging in *H. salinarum* NRC-1 UF during IR exposure. This is also supported by previous work showing the *H. salinarum* NRC-1 intracellular Mn/Fe ratio of 0.27 is much higher than both *E. coli*

and *P. putida* [34, 59] and the fact that there is a strong correlation between resistance to IR and Mn/Fe ratio [59].

*In vitro* studies have shown that Mn-phosphate complexes have a high capacity to scavenge hydrogen peroxide and superoxide [38]. Solutions containing 25μM Mn and 50mM $PO_4$ were capable of detoxifying superoxide produced via IR [43]. We found that the concentration of $PO_4$ was about five-fold more abundant in *E. coli* and *P. putida* UFs than *H. salinarum* NRC-1 UF. While this was surprising, the $PO_4$ was at mM concentrations while Mn was measured in μM concentrations, indicating that the limiting factor in formation of Mn-$PO_4$ complexes is the availability of Mn. *H. salinarum* NRC-1 contains up to 100 times the amount of Mn when compared to the other two UFs, indicating it would likely contain higher numbers of Mn-$PO_4$ complexes. Our data indicates that Mn-$PO_4$ complexes formed in *H. salinarum* NRC-1 UF may be critical for scavenging superoxide produced during IR exposure. One of the main cellular targets of superoxide is iron-sulfur groups of proteins [11], so detoxification of superoxide by Mn-$PO_4$ complexes will result in protection of proteins against oxidative damage.

In analysis of small molecules found in the UFs, we showed that the *H. salinarum* NRC-1 UF had slightly elevated levels of amino acids when compared to the radiation sensitive *E. coli* and *P. putida* UFs. However, it is unlikely that this result represents a significant accumulation of amino acids due to IR resistance because there is a wide variation in free amino acid concentration of UFs between the two

radiation sensitive organisms, *E. coli* and *P. putida*. Amino acid levels were probed because Mn-amino acid complexes have demonstrated detoxification of ROS in cell free lysates of *Saccharomyces cerevisiae* [91]. We then measured the total concentration of amino acids in the UFs to determine if short peptides were critical for scavenging of ROS produced by IR. Peptide chains in Mackeral hydrolysates show antioxidant properties, and the larger the chain, the greater the protection offered [108]. In addition, an 11-amino acid long peptide was isolated from microalgae that was capable of scavenging various ROS and protected DNA and survival of human cell lines from hydrogen peroxide exposure [109]. The increase between the free and total amino acids represents the number of amino acids that were bound up in short peptides in the UFs, and here we see similar increases for *H. salinarum* NRC-1, *E. coli*, and *P. putida* UFs. *D. radiodurans* UF showed a large increase in total versus free amino acids, indicating that there are significant amounts of amino acids combined into short peptides. These peptides may play a ROS scavenging role in *D. radiodurans*, however it is unlikely that scavenging by individual amino acids or short peptides is a major ROS scavenging systems employed by *H. salinarum* NRC-1.

The isocratic liquid chromatography separation of *H. salinarum* NRC-1 and *D. radiodurans* UFs revealed high levels of several nucleosides/nucleotides species that were not accumulated in *E. coli* UF. The peaks in *H. salinarum* NRC-1 UF had UV light absorption spectrums and elution times that were consistent with that of pyrimidines, possibly cytidine or uridine. The *D. radiodurans* UF also contained

species that could be pyrimidines, as well as some with elution times consistent with adenosine. These nucleosides may be critical for scavenging hydroxyl radicals that are produced during IR. *In vitro* assays have shown hydroxyl radical scavenging of adenosine and related nucleosides [110]. This is the first investigation of ROS scavenging of nucleosides *in vivo*. A possible mechanism in radiation resistant organisms, with higher Mn/Fe ratios, it may be that Mn binds to nucleosides to induce a site-specific reaction with hydroxyl radicals. Further work is being done to identify these nucleosides and determine their significance in protecting *H. salinarum* NRC-1.

This work investigated the role of *H. salinarum* NRC-1's non-enzymatic IR scavenging system in protecting cellular macromolecules from IR and determined the components involved in protein protection. During IR, *H. salinarum* NRC-1 UF showed a similar level of DNA protection as that of *E. coli* and *P. putida* but a remarkable increase in and protein activity protection over that of those two radiation sensitive bacteria. The high concentrations of halides present in *H. salinarum* NRC-1 provided limited protection to DNA but not to protein activity with IR. Biochemical analyses of the enzyme-free UFs revealed that $MnPO_4$ and high concentrations of nucleosides might be critical factors in scavenging ROS produced by IR in *H. salinarum* NRC-1. To further this research, the nucleosides accumulated in *H. salinarum* NRC-1 UF will be separated and identified using tandem mass spectroscopy. These individual nucleosides will be tested for their ability to protect enzyme function during IR exposure, possibly with the addition of Mn and $PO_4$ to the concentrations found in the *H. salinarum* NRC-1 UF. In addition, levels of individual

amino acids in the *H. salinarum* NRC-1 UF will be measured to determine if there is accumulation of any specific amino acids that could be critical for ROS scavenging.

# Chapter 5: Conclusions

The existence of organisms that are extremely resistant to ionizing radiation (IR) is unusual due to the lack of naturally occurring environments that have such high radiation levels. The fact that desiccation resistant organisms are often resistant to IR has led to the hypothesis that IR resistance is a by-product of adaptation to desiccating conditions and the accompanying oxidative stress [4-6]. To understand the extent of oxidative damage with IR in contrast with chemical oxidants, we first characterized damage to the cell's macromolecules introduced by IR, superoxide, and hydrogen peroxide. The three treatments showed distinct damage profiles in the radiation resistant archaeon *Halobacterium salinarum* NRC-1. The significantly higher level of DNA and protein damage with IR, for similar levels of cell survival, suggested that cell death with superoxide and hydrogen peroxide resulted from interference with major metabolic pathways rather than generalized oxidative lesions. We also showed that the positive correlation between protein oxidative damage and cell death previously established with IR [7] did not hold true for oxidative stress with hydrogen peroxide and superoxide. This, again, underlined the idea that more complex metabolic interactions are at play with chemical oxidative stress, which could be related to the location and the nature of the oxidative stress. In our experiments, superoxide stress was applied through exposure to paraquat, a redox cycling drug that produces superoxide by taking electrons from redox proteins involved in cellular respiration [11], possibly interfering with energy production. The different profiles for oxidative damage that we measured in *H. salinarum* NRC-1cells

were also reflected by the distinct stress responses of the organism at the transcriptional level when exposed to hydrogen peroxide, paraquat, and IR [26, 31]. Future work could include characterization of the damage profile and transcriptional response resulting from desiccation. If the effects of desiccation and IR were alike, this would strengthen the hypothesis that IR and desiccation introduce similar stresses to the cells and elucidate the underlying mechanisms for desiccation resistance in microorganisms, with implications for higher organisms.

Second, we characterized the roles of ROS scavenging enzymes in the protection of cellular macromolecules from the deleterious effects of chemical oxidants and IR. Gene deletion mutants of ROS scavenging enzymes were tested for survival, and levels of DNA and protein damage were measured following ROS treatment. While we found that most ROS scavenging enzymes were essential for cell survival to superoxide and hydrogen peroxide stress, there was no correlation between mutant survival and oxidative damage to DNA and protein, supporting further the idea that metabolic interference rather than generalized oxidative lesions is the cause of cell death with exposure to superoxide and hydrogen peroxide. Further knowledge could be gained through characterization of the deletion mutants' abilities to protect cellular macromolecules at different concentrations of chemical oxidants. Although the levels of oxidants used in this study correspond to 80% survival of the control strain, superoxide exposure (via paraquat) was found to elicit a much greater stress response than hydrogen peroxide [26]. Indeed, at sub-inhibitory levels of superoxide exposure (0.25mM paraquat), a different stress response was observed in *H. salinarum* NRC-1

[26]. Therefore, cells could be tested at a lower concentration of superoxide where the cellular response may be more focused on damage avoidance, and therefore these ROS scavenging enzymes might be more relevant in protection of DNA and proteins.

The finding that none of the ROS scavenging enzymes we tested were essential for *H. salinarum* NRC-1 survival to IR is novel and suggests that there may be other processes - potentially non-enzymatic - at work in protecting the cell's macromolecules against ROS produced during IR. Comparison of non-enzymatic ultrafiltrates (UF) from *H. salinarum* NRC-1 and the radiation sensitive *E. coli* and *P. putida* showed that *H. salinarum* NRC-1 UF was more adept at protection of protein function, but not at prevention of DNA strand breakage during IR exposure. These data support the idea that protein protection is critical to resistance to IR [7]. The individual components of this UF were identified and quantified to determine the source of *H. salinarum* NRC-1's remarkable protein protection. Halides Cl and Br were found to protect DNA and proteins from IR at levels too low to account for *H. salinarum* NRC-1 UF's protection abilities. Previous work showed that *H. salinarum* NRC-1 and other radiation resistant organisms have much higher intracellular Mn/Fe ratios than the radiation sensitive bacteria *E. coli* and *P. putida* [34]. This calls into question whether Mn in *H. salinarum* NRC-1 may be scavenging ROS as it has been demonstrated *in vivo* that $MnPO_4$ complexes can scavenge superoxide [43] and Mn-amino acid complexes are capable of the disproportionation of hydrogen peroxide [42]. *E. coli* and *P. putida* UFs contained more $PO_4$ than *H. salinarum* NRC-1 UF, however *H. salinarum* NRC-1 UF contained over 100 fold more Mn, suggesting that

the ROS scavenging activities in *H. salinarum* NRC-1 could be carried out by Mn-$PO_4$ complexes [43]. Using LC-MS we detected three nucleosides species in much greater abundance in *H. salinarum* NRC-1 UF as compared to *E. coli*, indicating that these molecules may play a significant role in scavenging hydrogen peroxide during IR exposure [110]. Indeed, *in vitro* data showed adenosine and related nucleosides were capable of scavenging hydroxyl radicals [110]. These finding indicate that *H. salinarum* NRC-1 contains non-enzymatic small molecule scavengers that might be critical for protein protection during IR.

It is currently hypothesized that *H. salinarum* NRC-1's IR resistance is due to its resistance to desiccation encountered in its natural environment [3]. There are a number of molecular features of *H. salinarum* NRC-1 that contribute to its IR resistance. This organism accumulates high levels of intracellular salts to balance the osmotic pressure of its environment, and these halides have shown evidence of limiting oxidative damage to DNA and proteins during IR exposure [9]. *Halobacterium salinarum* NRC-1 also contains up to 25 copies of its genome, meaning there are ample copies of the genome available for recombination repair to reconstruct its genome following fragmentation by IR [51]. There is also evidence for efficient DNA repair systems [10, 85]. *H. salinarum* NRC-1 contains a high intracellular Mn/Fe ratio [9], which is a feature correlated with IR resistance in prokaryotes [59]. Our findings here indicate that this Mn may be forming complexes with $PO_4$ to scavenge superoxide radicals produced in IR. In addition, specific

accumulated nucleosides in *H. salinarum* NRC-1 may scavenge hydrogen peroxide produced during IR and contribute to its radiation resistance.

While there has been a great deal of focus on non-enzymatic scavenging of ROS in IR resistant organisms, it is important to remember that IR resistance is likely the result of many physiological conditions and processes that are not yet well characterized [93]. Further work might include characterization of the roles of overabundant nucleosides in protection of proteins during IR exposure. In addition, the role of Mn in *H. salinarum* NRC-1's protection could be assessed through characterization of deletion mutants of genes related to Mn transactions in the cell. Knocking out the putative Mn transporter genes *zurA*, *zurM*, or *ycdH* could result in cells with decreased concentrations of Mn while knocking out the Mn transport autorepressor gene *sirR* could result in increased cellular Mn concentration [111]. Survival of the cells during IR could be analyzed, as well as the IR protection abilities of ultrafiltrates that are generated from these strains. In addition, the ultrafiltrates of other extremophiles found to be IR resistant, such as *Pyrococcus furiosus* [30] and *Thermococcus gammatolerans* [45] could be probed for non-enzymatic ROS scavenging capabilities.

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