ABSTRACT

Title of Document: SURVIVING IONIZING RADIATION: GENERAL STRESS RESPONSE AND MECHANISMS FOR THE PREVENTION AND REPAIR OF DNA DAMAGE IN *HALOBACTERIUM* SP. STR. NRC-1

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The effects of ionizing radiation on the extremely halophilic Archaeon *Halobacterium* sp. str. NRC-1 can be divided into three central themes: protection from oxidative damages, response to ionizing radiation, and repair of DNA double strand breaks (DSBs). Intracellular salts used to maintain osmotic balance in the hypersaline conditions *Halobacterium* cells require are shown in this study to provide *in vivo* protection from oxidative damages through the scavenging of hydroxyl radicals produced from the radiolysis of water by gamma radiation. These results highlight both the importance of the intracellular environment in determining radiation resistance and the multiplicity of pathways resulting in radiation resistance that can be utilized by various microbes resulting from their adaptations to common environmental stresses such as desiccation. The global stress response to gamma

radiation was measured using both genomic and proteomic methods. The resulting systems view reveals cooperation amongst several cellular processes including DNA repair, increased protein turnover, apparent shifts in metabolism to favor nucleotide biosynthesis and an overall effort to repair oxidative damage. Further, we demonstrate the importance of time dimension while correlating mRNA and protein levels and suggest that steady state comparisons may be misleading while assessing dynamics of genetic information processing across transcription and translation. The repair of DNA DSBs incurred after exposure to gamma radiation was examined in greater detail. The *in vivo* role of the Mre11/Rad50 complex was determined in an archaeal model system to determine if these proteins performed the same role in homologous recombination repair as their eukaryotic homologs. Deletion of *mre11* was found to reduce the rate of DSB repair, but not the overall survival of the cells. Taken together, the data presented here provide a halophilic model for radiation resistance that shares some common elements with other radiation resistant organisms such as *Deinococcus radiodurans* while presenting alternative mechanisms specific to extreme halophiles.

SURVIVING IONIZING RADIATION: GENERAL STRESS RESPONSE AND MECHANISMS FOR THE PREVENTION AND REPAIR OF DNA DAMAGE IN *HALOBACTERIUM* SP. STR. NRC-1

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Dedication

To my family and friends, who loved and supported me through the past decade of studying.

Acknowledgements

First of all, I'd like to thank Dr. Jocelyne DiRuggiero who, as my advisor, believed in me and helped me do the kind of work that I wanted to do. I am a better scientist because of all the hard work you have invested in me. Thanks is also required for the various lab members who have helped me on the projects included in this dissertation; Russell Rossenblatt, Molly Kottemann, Courtney Robinson, Katie Flanders, Courtney Busch (DiRuggiero Lab). A special thanks goes to my collaborators: Nitin Baliga and Kenia Whitehead (Institute for Systems Biology) for collaboration on transcriptional response to gamma irradiation in *Halobacterium*, Vince Adams and Alia Weaver (UMDCP Gamma Source Facility) for going above and beyond in their assistance in numerous gamma irradiations, Miral Dizdaroglu (NIST Mass Spectroscopy Facility) for DNA base oxidation analyses, and Michael Daly and lab members (USUHS) together with Jim Fredrickson and Shu-mei Li (PNNL) for ICP-MS analysis of intracellular Mn/Fe and use of the USUHS gamma facility.

Finally, I would like to extend my gratitude to my family for endless support. They listened to me describe my projects with great excitement, listened to the disappointments of failed experiments, and showed off printed copies of my published papers to anyone who would listen. Special thanks go to my mom for her practical support during my final push to graduation. I could not have done it without you. To all my friends, I promise to stop talking about science now…at least until the next project starts!

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List of Abbreviations

8-oxo-Gua, 8-hydroxyguanine
BER, base excision repair
DSB, double strand break
ESDSA, extended synthesis dependant strand annealing
Fapy-Ade, 4,6-diamino-5-formamidopyrimidine
Fapy-Gua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine
HR, homologous recombination
NER, nucleotide excision repair
NHEJ, non-homologous end joining
PFGE, pulsed-field gel electrophoresis
ROS, reactive oxygen species
SSA, single strand annealing
SSB, single strand break

Chapter 1: Introduction

Radiation resistant microorganisms have recently garnered a great deal of attention from scientists seeking to define the mechanisms underlying the survival abilities of these microbes. Adaptations enabling survival after exposure to levels of ionizing radiation not found in natural microbial habitats are thought to have arisen in response to repeated cycles of desiccation that produce the same damages to cellular macromolecules as ionizing radiation (Mattimore and Battista 1996). Ionizing radiation has therefore been proposed as a proxy source for oxidative damages induced by desiccation. Much of the focus has been on DNA repair mechanisms ranging from homologous recombination (HR) (Wyman et al. 2004; Dudas and Chovanec 2004; Kowalczykowski et al. 1994) to non-homologous end joining (NHEJ) (Dudasova et al. 2004) and synthesis-dependant strand annealing (Zahradka et al. 2006; Paques and Haber 1999; Paques and Haber 1997). Whole-genome studies of the transcriptional and translational responses of a variety of microorganisms from all three domains of life, including *Saccharomyces cerevisiae* (Birrell et al. 2002), *Deinococcus radiodurans* (Liu et al. 2003), and *Halobacterium* sp. str. NRC-1 (Whitehead et al. 2006), to ionizing radiation have been used to search for proteins and pathways responsible for radiation resistance. These investigations, however, have not revealed a 'silver bullet' repair protein or pathway that can explain the survival abilities of extremophiles. In addition, recent evidence suggests that prevention of protein oxidation also contributes to radiation resistance in prokaryotes with a high Mn/Fe ratio (Daly et al. 2004). Combined, the data provides tantalizing

evidence that radiation resistance may be the result of a complex set of intracellular reactions and molecular pathways which vary from one group of organisms to another, rather than simply the result of potent DNA repair abilities common to all extremophiles.

Archaea provide exceptional model systems for molecular-level investigations of microbial survival abilities. The Archaea domain is composed of prokaryotic microorganisms that are often – but not exclusively – extremophiles, including hyperthermophiles, methanogens and extreme halophiles (see Figure 1-1). Archaeal DNA metabolism proteins are more closely related to eukaryotic proteins than to the bacterial counterparts, with the notable difference that archaeal repair systems have fewer proteins involved than their eukaryotic counterparts, making them ideal for use in deciphering more complex eukaryotic pathways in a simplified setting. The extremely halophilic archaeon *Halobacterium* sp. str. NRC-1 has been shown to be highly resistant to both desiccation and gamma irradiation (Kottemann et al. 2005) (Whitehead et al. 2006). *Halobacterium* cells also maintain an intracellular salt environment in equimolar amounts to the external hypersaline environment, providing a distinct context for studying the intracellular reactions responsible for radiation resistance.

This review seeks to summarize the recent discoveries in the field of microbial radiation resistance from an archaeal perspective.

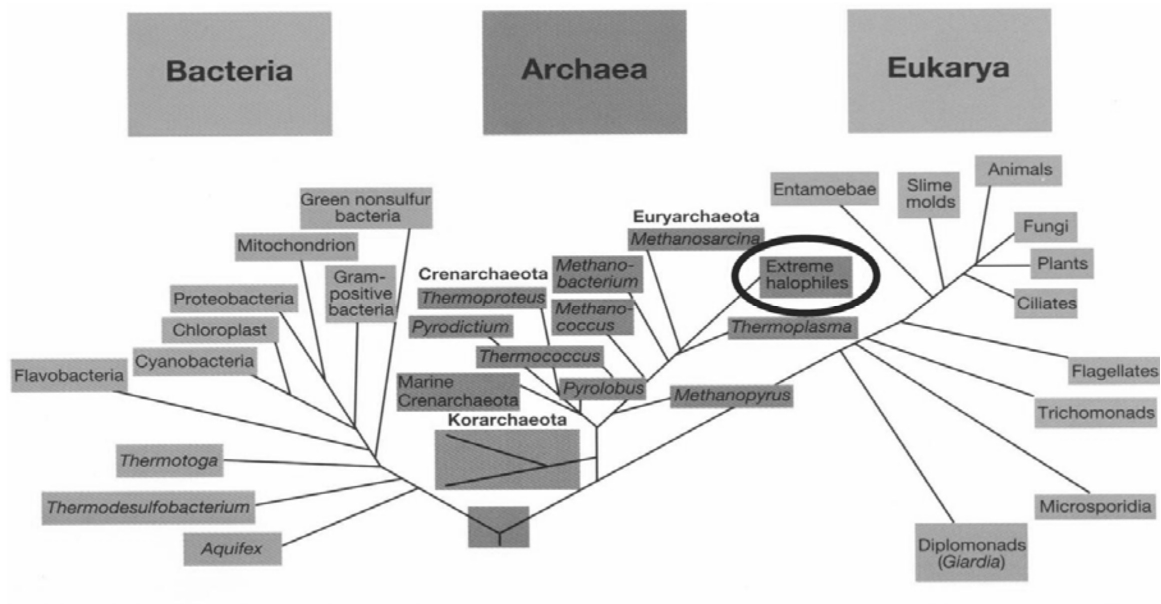


Figure 1-1. Universal tree of life showing the three domains (Bacteria, Eukarya, Archaea) taken from (M.T. 2000). The Archaea are divided into two major kingdoms (Crenarchaeota and Euryarchaeota) along with the Korarchaeota, composed of thermophilic archaea enriched from environmental samples, and the Nanoarchaeota (not shown here). The extreme halophiles, of which *Halobacterium* sp. str. NRC-1 is a member, are circled.

Ionizing Radiation and Oxidative Damage

Ionizing radiation is a well-characterized exogenous source of free radicals produced via the radiolysis of water, which account for more than 80% of the DNA damage whereas less than 20% is the result of direct effects of photons (Riley 1994). Aerobic organisms also produce reactive oxygen species (ROS) endogenously through the autooxidation of dehydrogenases involved in the respiratory electron transport chain (reviewed in Imlay 2003). ROS are able to attack and modify DNA bases as well as sugar moieties, proteins, and lipids (Riley 1994).

The macromolecular targets and activities vary between reactive oxygen species. H_2O_2 is not highly reactive, but unlike superoxide and hydroxyl radicals, it is membrane permeable. It can also be converted to more reactive species through both the Fenton and Haber-Weiss reaction pathways (see review in (Valko et al. 2005)).

Fenton reaction:

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

Haber-Weiss reaction:

$H_2O_2 + {}^{\cdot}OH \rightarrow H_2O + O_2^- + H^+$

$H_2O_2 + O_2^- \rightarrow O_2 + OH^- + {}^{\cdot}OH$

Superoxide is able to oxidize iron-sulfur clusters of proteins, destabilizing the protein structure and causing the release of free Fe(II), which can then combine with H_2O_2 to

produce the biologically damaging hydroxyl radical ($^{\cdot}OH$) via the Fenton and Haber-Weiss reactions. The reduction of free iron is a rate-limiting step for DNA oxidative damage (Park and Imlay 2003). The hydroxyl radical is highly reactive and can modify proteins, lipids, and nucleic acids. Oxidation of sugar and base components of DNA by hydroxyl radicals produces bulky lesions (Cadet et al. 1999; Hutchinson 1985; Dizdaroglu et al. 2002) which can stall replication forks (Michel 2000). The most common oxidization products are 7,8-dihydro-8-oxoguanine (8-oxoguanine) for purines and thymine glycol for pyrimidines (Slupphaug et al. 2003).

Much attention has been focused on the cytotoxic effects of DNA oxidation by free radical species, emphasizing the critical nature of DNA repair systems for radiation resistance (Daly and Minton 1995; Billi et al. 2000; Bennett et al. 2001). Recent reports, however, have suggested that loss of protein function due to protein oxidation may be more significant in terms of cell survival (Daly et al. 2007). In light of the potentially lethal effects of exposure to ionizing radiation including arrested DNA replication, chromosomal fragmentation, and loss of protein function, mechanisms to protect against or repair the damages incurred upon exposure to ionizing radiation are vital to cell survival.

Protective Strategies against Ionizing Radiation

A number of protective mechanisms have been noted for shielding DNA from radiation damages. Sensory rhodopsin pigments in *Halobacterium* cells are responsible for initiating a phototaxis response away from high energy wavelengths

(DasSarma et al. 2001). C_{50} carotenoids found in the cell membranes of both *Halobacterium* (Kottemann et al. 2005) and *D. radiodurans* (Carbonneau et al. 1989) have been shown to offer DNA protection from oxidative damages, particularly at low doses of ionizing radiation, through the scavenging of hydroxyl radicals. Superoxide dismutases, along with catalase and peroxidase enzymes, scavenge superoxide and hydrogen peroxide radicals, respectively (Aguirre et al. 2005; Cannio et al. 2000; Keyer et al. 1995).

Abiotic mechanisms have been noted for the protection of cellular macromolecules. Iron compounds have been shown to be effective in shielding DNA against UV radiation (Cockell and Knowland 1999). $MgCl_2$ and KCl have been shown to protect DNA from thermodegradation *in vitro* (Marguet and Forterre 1998). Intracellular KCl has also been hypothesized to act as a protective agent against oxidative damage by scavenging of the hydroxyl radical by chloride ions (Kottemann et al. 2005; Shahmohammadi et al. 1998). Iron cofactors in many cellular proteins including some forms of superoxide dismutase have long been known to react with hydrogen peroxide increasing the production of the highly reactive hydroxyl radical through Fenton chemistry. Mn(II) can be used in place of Fe(II) as a cofactor for many enzymes including superoxide dismutase, and Mn (II) does not participate in Fenton chemistry. Recent data has shown that many radiation resistant bacteria have a higher intracellular Mn/Fe ratio than radiation sensitive organisms (Daly et al. 2004). This mechanism was hypothesized to reduce oxidative damage to intracellular proteins by

superoxide radicals, thus preserving the DNA repair functions of the cells during periods of oxidative stress (Daly et al. 2007).

DNA Repair Strategies after Exposure to Ionizing Radiation

Repair of oxidative damages to DNA can be achieved by multiple repair pathways, including base excision repair (BER), nucleotide excision repair, translesion synthesis, synthesis dependant strand annealing (SSA), non-homologous end joining (NHEJ), and homologous recombination (HR), working in concert on the various damage types (Swanson et al. 1999; Slupphaug et al. 2003). The most commonly employed mechanisms are BER for the removal of oxidized DNA bases, and SSA, NHEJ, and HR for the repair of DNA double strand breaks (DSBs).

The BER pathway utilizes lesion-specific glycosylases (see review in (Dizdaroglu 2003)), such as the *Escherichia coli* Fpg and endo III enzymes, to recognize and excise oxidized DNA bases, followed by cleavage of the phosphate backbone on either the 5’ side of the AP site by an AP endonuclease or on the 3’ side by an AP lyase. DNA synthesis is followed by ligation of the phosphate backbone by DNA ligase. Oxidative damage to DNA bases and sugar moieties, however, often occur in clusters within 2 helical turns of the DNA on opposite strands resulting in the formation of DSBs during attempted base excision repair (Blaisdell and Wallace 2001b; Dianov et al. 2001; Blaisdell et al. 2001).

Repair of DNA DSBs is carried out by SSA, NHEJ, and HR pathways. Various forms of single strand annealing (SSA) have been proposed for the repair of DNA DSBs including inter-chromosomal SSA (Daly and Minton 1996) and intra-chromosomal SSA (Paques and Haber 1999), synthesis dependant strand annealing (Paques and Haber 1999), and extended synthesis dependant strand annealing which involves the formation of crossover structures by a RecA-dependant homologous recombination process (Zahradka et al. 2006). Inter-chromosomal SSA has been shown to be involved in DNA DSB repair in *D. radiodurans* only within the first few hours after exposure to ionizing radiation, after which the HR pathway is activated (Daly and Minton 1996).

NHEJ tends to be favored in G_0/G_1 phases of the cell cycle in *S. cerevisiae*, and has been shown to precede HR (reviewed in Sonoda et al. 2006), although the HR pathway is most heavily utilized overall (Aylon and Kupiec 2004). NHEJ requires no region of homology between chromosomal fragments, resulting in an increased potential for mutagenesis. The NHEJ pathway involves the use of the Ku70 and Ku80 proteins as a heterodimer together in a complex with a DNA-dependant protein kinase for DSB recognition, followed by recruitment of the Mre11/Rad50/Xrs2 complex prior to DNA end processing and ligation of the break by a DNA ligase. Much of this process is currently undefined, including the order of activity, and the role of the Mre11/Rad50/Xrs2 complex, which is required for this process in *S. cerevisiae*. The common feature among all organisms currently shown to be capable of NHEJ is the presence of Ku70 and Ku80 homologs (Aylon and Kupiec 2004). NHEJ has been

shown to function as a back-up system when the HR pathway is disrupted (reviewed in van Gent et al. 2001)

The process of HR repair of DNA DSBs in both Bacteria and Eukarya has been extensively studied (see reviews in (Symington 2002; Krogh and Symington 2004; Wyman et al. 2004; Kowalczykowski et al. 1994; West 2003)). HR is used preferentially in S/G_2 phases in yeast due to the presence of sister-chromatids for use as templates, which greatly reduces the potential for mutagenesis compared with NHEJ. The HR pathway is utilized by organisms in all three domains of life.

The maintenance of genome integrity and fidelity is critical for cell survival after exposure to ionizing radiation, requiring the repair of chromosomal fragmentation resulting both from the action of ROS on DNA components as well as strand breaks produced during the repair of clustered DNA damages. Owing to the universality of the HR repair pathway, and the importance of DNA DSB repair to cellular survival after exposure to ionizing radiation, this pathway will be investigated here in greater detail.

Homologous Recombination Repair of DNA DSBs in the Three Domains of Life

There are five basic steps to homologous recombination (HR) repair of DNA DSBs: (1) DSB recognition and end processing to create 3'-OH overhangs which are required for recombinase recognition, (2) recombinase loading, (3) homologous

strand invasion by the recombinase forming a Holliday junction, (4) Holliday junction branch migration and DNA synthesis, and (5) Holliday junction resolution. The general HR pathway is outlined in Figure 1-2, and the functional homologs from model species in each of the three domains of life are given in Table 1-1. The structures of the proteins responsible for these activities are different in the Bacteria and the Eukarya, as well as some of the specific activities, but the general process is the same.

Homologous recombination is currently best characterized in the Bacteria (reviewed in Kowalczykowski et al. 1994). *E. coli* utilizes the RecBCD, RecA, RuvAB, and RuvC proteins for DSB detection and end processing, homologous strand invasion, branch migration, and Holliday junction resolution, respectively. The RecBCD enzyme binds to DNA DSBs, and uses the enzyme's helicase activity to unwind the DNA until a χ (chi) site is encountered while using the enzyme's ATP-dependant nuclease activity to degrade the 3' strand. Location of a χ site signals a stop to the strong 3'-5' nuclease function with a nicking of the DNA 3' to the χ site while the helicase activity continues. The nuclease activity switches to a weak ssDNA 5'-3' nuclease degrading the 5' strand producing a 3' ssDNA overhang coated in single strand binding protein (SSB) with the χ site near its terminus. A nucleoprotein

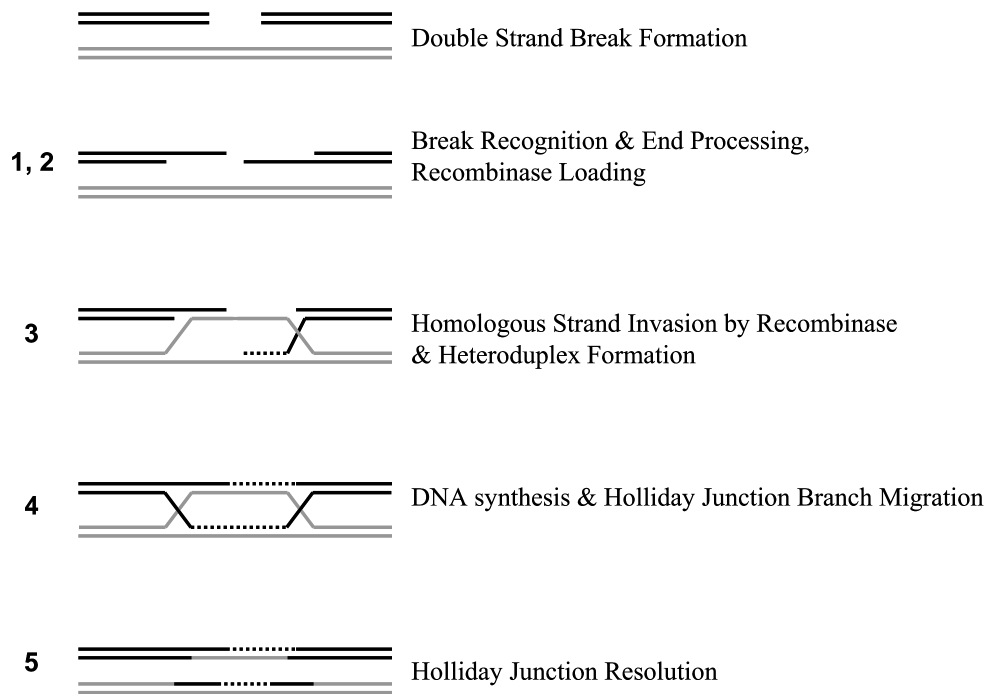


Figure 1-2. General model of DNA DSB repair via the homologous recombination pathway showing the five major steps: (1) DSB recognition and end processing to create 3'-OH overhangs which are required for recombinase recognition, (2) recombinase loading, (3) homologous strand invasion by the recombinase forming a Holliday junction, (4) Holliday junction branch migration and DNA synthesis, and (5) Holliday junction resolution.

	Bacteria [*E. coli*]	**Eukarya [*S.cerevisiae*]**	**Archaea [*Halobacterium*]**
DNA DSB recognition and end processing to create 3'-OH overhang	RecBCD	Mre11/Rad50/Xrs2	Mre11, Rad50, ?
Recombinase loading	RecBCD	Rad52, Rad54/54/57	Mre11, Rad50 (?)
Homologous strand invasion (recombinase)	RecA	Rad51	RadA (also called RadA1)
Srand exchange	RecA, SSB	Rad51,Rad52, Rad54/54/57, RPA	RadA, RPA
Holliday junction branch migration	RuvAB	Rad54 (?)	Hjm
Holliday junction resolution	RuvC	?	Hjr

Table 1-1. Homologous recombination repair functional homologs in the three domains of life. The proteins listed are members of the most commonly utilized pathway in cases where more than one pathway is available (eg. RecBCD and RecFOR pathways in *E. coli*). Archaeal proteins have been characterized *in vitro*, usually using proteins from *Pyrococcus furiosus* and *Sulfolobus solfataricus*. Homologs based on sequence similarity in *Halobacterium* are given. Archaeal homologs of Hjr include Hjc in *P. furiosus* and both Hjc and Hje in *S. solfataricus*.

filament composed of monomers of the RecA recombinase forms around the ssDNA while initiating strand exchange with a homologous region of DNA. A Holliday junction is formed providing templates for DNA synthesis in both strands. The RuvAB proteins increase the branch migration activity of the RecA filament until the Holliday junction is resolved using the RuvC endonuclease. An alternative pathway nominally used to repair single strand gaps in dsDNA involves the use of the RecFOR proteins in place of RecBCD for recruitment of RecA onto ssDNA coated in SSB. The use of the RecQ helicase and RecJ 5'-3' exonuclease can convert this into a DSB repair pathway.

The HR pathway in eukaryotes is more complex, involving an increased number of proteins. The proteins responsible for the main functions in the HR pathway have been reviewed (Aylon and Kupiec 2004; Symington 2002; Krogh and Symington 2004; West 2003; Wyman et al. 2004; Paques and Haber 1999), and are shown in Table 1-1. The Mre11/Rad50/Xrs2 complex in *S. cerevisiae* functions in DSB identification and end processing. No χ – like site is required in the eukaryotic HR pathway. RPA is the functional homolog of bacterial SSB for ssDNA protection and recognition by Rad51, the eukaryotic homolog of RecA. Rad52 is responsible for recombinase loading, and together with Rad54, Rad55, and Rad57 is involved in promoting Rad51 strand exchange activities. Currently unknown in the eukaryotic pathway are the proteins involved in Holliday junction resolution for nuclear DNA

(Heyer et al. 2003), although recent evidence suggests that Rad54 may act in Holliday junction branch migration (Bugreev et al. 2006).

Gaps in knowledge of the proteins responsible for branch migration and Holliday junction resolution in the eukaryotic HR pathway have proven difficult to fill (Heyer et al. 2003). Proteins responsible for these processes have been identified in archaeal model systems. Archaea, despite having circular chromosomes contained within prokaryotic cells, possess DNA processing proteins most closely related to eukaryotic proteins and can serve as simplified models of DNA repair and replication mechanisms found in these more derived systems. Archaea utilize eukaryotic-like Orc/Cdc6 and MCM replication initiation proteins, as well as PCNA sliding clamp for replication, TATA-boxes bound by multiple TATA-binding protein and TFB transcription factors. DNA repair proteins are generally more similar to their eukaryotic homologs than to bacterial proteins.

Proteins for all the major processes in the HR pathway have been identified in the Archaea. Rad50 and Mre11 proteins from *Pyrococcus furio*sus have been characterized *in vitro* and their 3-dimensional structure determined (Hopfner et al. 2001; Hopfner et al. 2002; Hopfner et al. 2000a; Hopfner et al. 2000b). The structure of the RadA archaeal recombinase has been resolved from both a thermophile, *Sulfolobus solfataricus* (Ariza et al. 2005), and a methanogen, *Methanococcus voltae* (Wu et al. 2004), as well as characterized biochemically in a number of thermophilic species including *P. furiosus* (Seitz and Kowalczykowski 2000), and characterized

genetically in the haloterant *Haloferax volcanii* (Woods and Dyall-Smith 1997). Proteins responsible for branch migration and Holliday junction resolution activities lacking in Eukaryotic systems have been identified in archaeal hyperthermophilic species. The Hjm branch migration protein has been characterized *in vitro* (Fujikane et al. 2006), as has the structure-specific Hef helicase/endonuclease (Komori et al. 2002; Komori et al. 2004). Crystal structures have been derived for the Hef protein in both *P. furiosus* (Nishino et al. 2005; Komori et al. 2002) and *S. solfataricus* (Roberts and White 2005) revealing a DEAH-family helicase domain attached to a XPF-type nuclease domain functioning as part of a homodimer, whereas the Eukaryotic XPF and Mus81 proteins are found as separate heterodimer proteins. The structure of the Holliday junction resolvase in the Archaea has been determined from homologs in multiple organisms, including Hjc in *P. furiosus* (Nishino et al. 2001) and *S. solfataricus* (Middleton et al. 2003), the Hje paralog from *S. solfataricus* (Bond et al. 2001), and Hjc from *Archaeoglobus fulgidus* (Biertumpfel et al. 2005). Biochemical analyses of archaeal resolvases have shown these proteins to be functional homologs of the bacterial RuvC nuclease (Komori et al. 2000b; Komori et al. 2000c; Komori et al. 1999; Daiyasu et al. 2000) .

The *in vitro* biochemical analyses of archaeal HR proteins require further examination using genetic methods to determine their *in vivo* activities. There are, however, presently no practical genetic systems for hyperthermophiles such as *P. furiosus* that grows at 100°C, although a genetic system has recently been developed for *Thermococcus kodakaraensis* growing optimally at 85°C (Sato et al. 2005; Sato et

al. 2003). The genome of *Halobacterium* contains homologs of the major HR repair proteins identified in *P. furiosus*. In addition, the *Halobacterium* genome contains genes coding for proteins present in all major DNA repair pathways found in both the Bacteria and the Eukarya (Ng et al. 2000) with the exception of NHEJ, suggesting a major role for DNA repair in *Halobacterium* resistances to extreme conditions. Genomic systems readily available for *Halobacterium* allow for further examinations of the gene expression patterns for each of these genes as well as their regulation in response to stimuli such as ionizing radiation.

Halobacterium sp. str. NRC-1

Halobacterium is a member of the Euryarchaeota, one of the two kingdoms of the archaeal domain along with the Crenarchaeota, which includes methanogenic archaea as well as some hyperthermophilic species (see Figure 1-1). The Euryarchaeota are distinguished from the Crenarchaeota on the basis of their separate branching order in phylogenetic analyses of 16S rDNA sequences as well as differences in DNA replication machinery including the ssDNA binding and DNA polymerase proteins. *Halobacterium* is a useful model system under development for DNA repair studies due to the presence of genetic systems including shuttle vectors and targeted gene replacement/over-expression systems lacking in many other archaea including *P. furiosus*. The *Halobacterium* genome was sequenced in 2000 (Ng et al. 2000) and was found to consist of a GC-rich major chromosome (68% GC) and two mini-replicons; pNRC100 (58% GC) and pNRC200 (59% GC). As with other desiccation resistant microrganisms, *Halobacterium* cells contain multiple copies of their genome

in each cell. *Halobacterium salinarum* cells were found to have an average of 25 copies of the major chromosome in mid-log phase which was reduced to 15 copies in stationary phase (Breuert et al. 2006). This is considerably higher than the 8 genome copies found in mid-log phase of *D. radiodurans* cells (Hansen 1978). The presence of multiple genome copies is advantageous for homologous recombination repair of extensive DNA double strand breaks formed under desiccating conditions or ionizing radiation, providing a large number of DNA templates for recombination, although not a diagnostic of radiation resistant organisms as some prokaryotes with multiple genome copies are radiation-sensitive (see (Makarova et al. 2001) and references therein). *Halobacterium* genome was predicted to include 2,630 protein coding regions with 64% displaying sequence homology to genes in other organisms (Ng et al. 2000). A whole-genome oligonucleotide microarray for mRNA expression levels is available for *Halobacterium,* which is currently unavailable for other model systems such as *Haloferax volcanii*.

Hypersaline environments are characterized by elevated temperatures, fluctuations in oxygen and nutrient concentrations, high levels of solar radiation, and periodic desiccation (DasSarma and Fleischmann 1995). *Halobacterium* has been shown to be highly resistant to UV-C irradiation (D_{10}=340J/m^2 (Baliga et al. 2004)) as well as to both desiccation and gamma irradiation (D_{10}=5kGy) (Kottemann et al. 2005). Repair of extensive DNA DSBs produced both by desiccation and by gamma irradiation has been demonstrated within hours of damage formation in *Halobacterium* (Kottemann et al. 2005), as well as P. *furiosus* (DiRuggiero et al. 1997), and *D. radiodurans*

(Minton and Daly 1995). Gamma radiation resistance in *Halobacterium* is dependent on growth stage, with cultures in log phase exhibiting higher resistance than those in stationary phase (Kottemann et al. 2005). No inducible response similar to the SOS system in *E. coli* was observed (Kottemann et al. 2005). Halophilic microorganisms achieve osmotic balance in near-saturating salt environments through the accumulation of compatible organic solutes or by establishing an internal ionic environment equal in concentration, but not necessarily composition, to the extracellular environment (Da Costa et al. 1998). The cell interior of *Halobacterium* cells sequesters potassium and chloride ions while excluding sodium ions at molar concentrations equal to the cell exterior (Engel and Catchpole 2005). The high ionic strength of the *Halobacterium* intracellular environment compared to other radiation resistant organisms such as *D. radiodurans* provides an alternative framework from which to study abiotic mechanisms of radiation resistance.

Relevance to Astrobiology

Halobacterium inhabits an environment similar to that thought to have existed shortly after the Late Heavy Bombardment period approximately 3.5 billion years ago (McKay 1997). Evidence of halite within fluid inclusions in the Zag (Whitby et al. 2000) and Monahans (Zolensky et al. 1999) meteorites as well as on the surface of Mars (Rieder et al. 2004) highlights the importance of brine environments in the solar system, particularly as a source of liquid water in low-temperature environments. Halophiles are capable of surviving extended periods of desiccation trapped inside brine inclusions within salt crystals, although the exact time scale is currently being

debated (Vreeland et al. 2000; Nickle et al. 2002; Gruber et al. 2004; McGenity et al. 2000; Vreeland 2007). This has served to increase speculation on the existence of halobacteria on Mars (Landis 2001). Survival of halophiles on the Martian surface would require the ability to survive periodic desiccation as well as intense UV irradiation (Cockell et al. 2000) (Patel et al. 2004) and ionizing radiation. The use of model organisms such as *Halobacterium* for molecular-level studies into DNA repair and survival under extreme brine conditions is important to gaining insight into the potential for life on Mars.

Summary and Unresolved Questions in Microbial Radiation Resistance

There are many unresolved issues concerning the mechanisms underlying radiation resistance in microorganisms. The effect of the intracellular environment on production of free radical species and subsequent damage to macromolecules has not been fully explored. The relative toxicity of oxidative damage to DNA and proteins is currently being debated. Key proteins in the eukaryotic HR repair pathway, including proteins involved in branch migration and resolution of Holliday junctions, remain elusive, as does the precise role of the Mre11/Rad50 complex in DSB recognition and end processing. These are all areas that can be addressed using *Halobacterium* as a model archaeal system.

Research Objectives

The overall objective of this research is to use *in vitro* and *in vivo* approaches to elucidate the mechanisms used by *Halobacterium* cells to prevent, respond to, and repair oxidative damages to DNA after exposure to ionizing radiation.

The specific aims of this research were as follows:

1. Investigate the role of salts in prevention of DNA damage, DSBs and oxidized products, by ionizing radiation in *Halobacterium*.
2. Identify the genes and proteins that participate in the oxidative damage response in *Halobacterium* after exposure to ionizing radiation using genomic and genetic methods.
3. Characterize the cellular role of the Rad50 and Mre11 proteins in *Halobacterium* using genetic methods. The Rad50/Mre11 complex known to be involved in the homologous recombination DNA DSB repair pathway in the Eukaryotes.

The first significant contribution of this research was to demonstrate the role of intracellular salt in protection against DNA oxidative damages following exposure to ionizing radiation. This is a unique feature of extreme halophiles that contributes to their survival after irradiation. Secondly, this work presents an extensive picture of the transcriptional and translational response to damages induced by gamma irradiation, thereby providing a solid foundation of information upon which to initiate further investigations into the pathways responsible for radiation resistance. Thirdly,

in vivo studies into the homologous recombination pathway for DNA DSB repair pathway in *Halobacterium* presented here complement *in vitro* assays previously conducted using other archaea to determine the identity and function of the key pathway constituents in the third domain of life, and highlight the role of DNA oxidative damage repair in radiation resistance.

Chapter 2: Alternative Mechanisms for Radiation Resistance in the Halophilic Archaeon, *Halobacterium* sp. str. NRC-1

Introduction

The underlying causes of the observed cytotoxicity of ionizing radiation in micro-organisms have been a subject of increased investigation. Links have been made between survival mechanisms for desiccation and ionizing radiation based on the types of damage incurred by cellular macromolecules after exposure to both conditions (Mattimore and Battista 1996). DNA double strand breaks (DSBs) have been the focal point of many studies investigating the source of radiation resistance in microorganisms such as *Deinococcus radiodurans* (Daly and Minton 1995; Daly et al. 2004; Daly et al. 2007; Daly 2006; Battista et al. 1999;Zahradka et al. 2006). Genomic comparisons between radiation resistant prokaryotes have failed to reveal unique repair systems that would account for the survival of these microorganisms (Daly et al. 2007). Recent evidence has shown that prevention of protein oxidation also contributes to radiation resistance in prokaryotes with a high intracellular Mn/Fe ratio (Daly et al. 2004). This suggest that while DNA strand breaks have been propsed as the most obvious cause of the cytotoxic effect of ionizing radiation, oxidative damage to other cellular macromolecules such as proteins may play a larger role in cellular survival. It also suggests that the intracellular milieu has a great effect on the level and type of oxidative damage incurred by both DNA and proteins.

The main source of damage to DNA by ionizing radiation comes from the production of ROS through the radiolysis of water and it accounts for 70% of lesions (Blaisdell et al. 2001). Oxidation of DNA bases and sugar moieties, abasic sites, strand breaks, and cross-links to proteins are the major DNA damage from ionizing radiation, often producing complex clustered lesions, and resulting in DNA double strand breaks (DSBs) from attempted repair (Dianov et al. 2001; Regulus et al. 2007). Protein oxidation is also prevalent after exposure to ionizing radiation, resulting in the addition of carbonyl groups to protein residues (Imlay 2003). Oxidative damage to DNA disrupts DNA replication, particularly when combined with oxidation and inactivation of DNA replication and repair proteins, leading to cell death. Formation of DSBs in microorganisms has been observed under both desiccating conditions and gamma (γ) irradiation (DiRuggiero et al. 1999; Mattimore and Battista 1996), leading to the hypothesis that adaptations to desiccating conditions by microorganisms such as *D. radiodurans* and *Halobacterium* sp. str. NRC-1 enable their survival after exposure to ionizing radiation (Mattimore and Battista 1996). Ionizing radiation has therefore been used as a proxy source for oxidative damage induced by desiccation.

Protective mechanisms against the effects of ionizing radiation have been demonstrated at the molecular level. Bacterioruberin, a C_{50} carotenoid pigment, has been shown to offer protection against γ-radiation both *in vitro,* reducing DNA strand breaks at low doses (30Gy) (Asgarani et al. 1999) and thymidine degradation at high doses (12kGy) (Saito et al. 1997), and i*n vivo,* evidenced as decreased survival in

colorless mutants of both *Halobacterium salinarium* (Shahmohammadi et al. 1998) and *Halobacterium* sp. str. NRC-1 (Kottemann et al. 2005) irradiated with 600Gy and 2.5kGy of γ-irradiation, respectively. Scavenging of hydroxyl radicals by chloride ions has been previously tested *in vitro* using low doses of both UV ($30J/m^2$) (Asgarani et al. 1999) and γ–radiation (200Gy) (Shahmohammadi et al. 1998), showing the protective effect of 2M KCl for plasmid DNA. Intracellular salts such as KCl have been hypothesized to have a role *in vivo* for extreme halophiles that sequester intracellular salts to maintain osmotic balance (Kottemann et al. 2005). This suggests that the hypersaline environment in which *Halobacterium* flourishes may be a fundamental factor in its resistance to both desiccation and ionizing radiation.

Halobacterium sp. str. NRC-1 (*Halobacterium)* is an extreme halophile, requiring 3.5 to 5M NaCl (4.3M NaCl optimal) for growth. Halophiles can maintain osmotic balance by either using compatible solutes, thereby keeping the salts in the extracellular environment out while retaining water, or by establishing an intracellular salt concentration equal to that of the extracellular environment. *Halobacterium* cells follow the later strategy, sequestering potassium and chloride while eliminating sodium, resulting in an intracellular environment equal in concentration, but not composition, to the surrounding hypersaline pool (Engel and Catchpole 2005). The membrane protein responsible for pumping halides into *Halobacterium* cells is halorhodopsin, a light-activated proton pump (Kolbe et al. 2000) which has been shown to transport chloride and bromide ions with equal efficiency (Steiner et al. 1984). We have shown that *Halobacterium* is highly resistant to UV and ionizing

radiation and to desiccation (Kottemann et al. 2005). The free-radical scavenging capability of membrane pigments, specifically bacterioruberin, provided *Halobacterium* with protection against cellular damages by ionizing radiation (Shahmohammadi et al. 1998; Kottemann et al. 2005). Intracellular high salt concentration was also found to provide a protective environment against DNA damage over a range of radiation doses by decreasing the number of DNA DSBs formed following γ-irradiation (Kottemann et al. 2005). Whole genome transcriptional analysis revealed that HR might be the major pathway for the repair of DSBs in *Halobacterium* and homologs of eukaryotic HR proteins have been identified in its genome (Whitehead et al. 2006; Ng et al. 2000).

To date, the hypothesis that the scavenging of hydroxyl radicals by intracellular chloride ions in extreme halophiles such as *Halobacterium* is a significant factor in the radiation resistance of these species has not been tested *in vivo*. We show here evidence that hydroxyl radical scavenging by halides offers significant protection to DNA damage that include base oxidation and DSBs and to oxidation of protein residues, using *Halobacterium* sp. str. NRC-1 as a model system. This work is also the first to report the extent of oxidative base damage in a prokaryotic organism after γ–irradiation - quantified by detecting base oxidation using GC/MS – underscoring the relative importance of oxidative damage to DNA other than strand breakage.

Results

We used the model halophile *Halobacterium* sp. str. NRC-1 to investigate the scavenging effect of hydroxyl radicals, produced by γ-irradiation, by intracellular halides in extreme halophiles. *Halobacterium*, and a majority of extreme halophiles, use KCl as the major compatible solute to counterbalance the high salinity of their natural environment. We determined the level of oxidative damage to DNA bases, DNA backbone and protein residues after exposure to ionizing radiation both *in vitro* and *in vivo.*

In Vitro DNA Protection against Oxidative Damage by Halides

Plasmid pUC19 DNA in a 1mM potassium phosphate buffer solution (pH 7.2), with and without addition of salts (2M and 4M KCl or KBr), was exposed to 0-300Gy of γ-radiation. After dialysis against the buffer solution, the DNA was analyzed by agarose gel electrophoresis to separate the damaged open circular form plasmid DNA from the undamaged supercoiled form. Opposing DNA single strand breaks (SSBs) in close proximity are thought to be responsible for observed double strand breaks on genomic DNA using electrophoresis methods (Cox and Battista 2005). SSBs stabilized by proteins or conditions are converted into DSBs during gel electrophoresis thereby potentially mis-representing the *in situ* condition. The assay used here detects DNA SSBs incurred after exposure to γ-radiation as a reduction in plasmid supercoiling. Figure 2-1 shows that KCl at or below biologically relevant

intracellular concentrations in *Halobacterium* cells (4M) has a protective effect for DNA exposed to ionizing radiation. At a dose of 300Gy, we found close to 2-fold fewer DNA strand breaks when KCl was added to the buffer before irradiation. The protection against DNA strand breaks by KBr is even greater, with 2.6-fold fewer breaks when 2M or 4M KBr were added to the buffer. Interestingly, the salt protection effect did not increase when the salt concentration was increase from 2M to 4M in the buffer.

Halobacterium Growth and Ionizing Radiation Resistance in Altered Salt Media

The survival of *Halobacterium* to γ-irradiation was investigated with cells grown at three NaCl concentrations, low (3.4M), optimal (4.3M; standard GN101 medium) and high (5.1M). Higher or lower salt concentrations than those inhibited cell growth and therefore were not used in this experiment. Alteration of the salt concentration in the growth medium from 3.4 to 5.1 M NaCl did not result in significant change in the survival of *Halobacterium* to γ-irradiation (Figure 2-2A). We also tested the protective effect of bromide over chloride *in vivo* by irradiating *Halobacterium* cells grown in medium where NaCl was replaced with increasing concentrations of sodium bromide. Cultures failed to grow with total replacement of NaCl by NaBr but showed significant growth up to 2.6M NaCl replaced by NaBr (data not shown). Again, no significant change in radiation resistance was detected at any concentration of NaBr (Figure 2-2B).

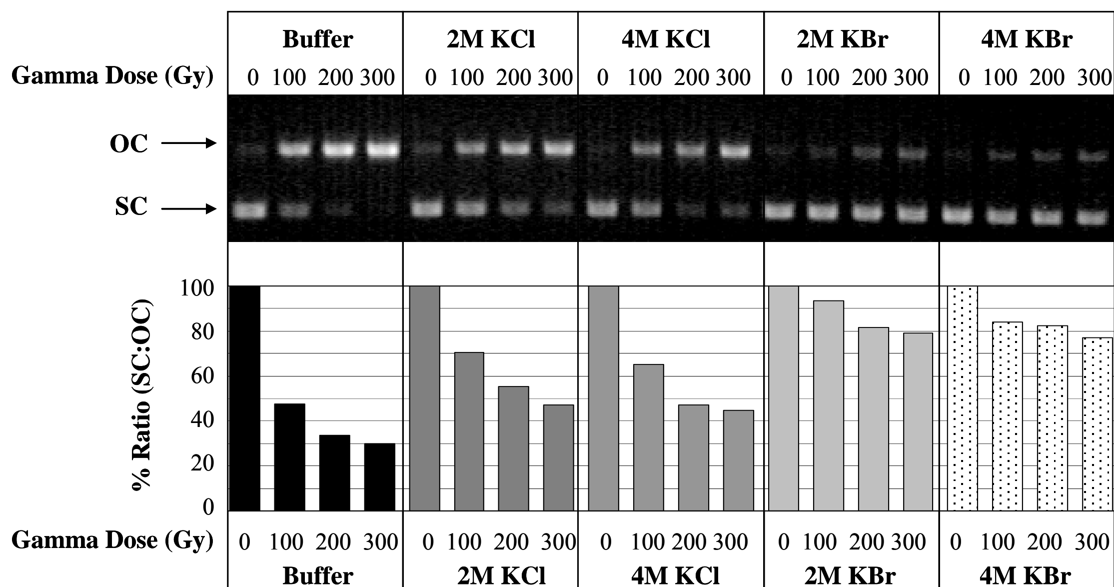


Figure 2-1. Agarose gel electrophoresis of pUC19 plasmid DNA after exposure to up to 300Gy of γ–radiation (top panel) showing both undamaged supercoiled (SC) and damaged open circular (OC) forms of DNA. Percent ratio of supercoiled to open circular form (bottom panel) was quantified by measuring the fluorescent intensity of each band.

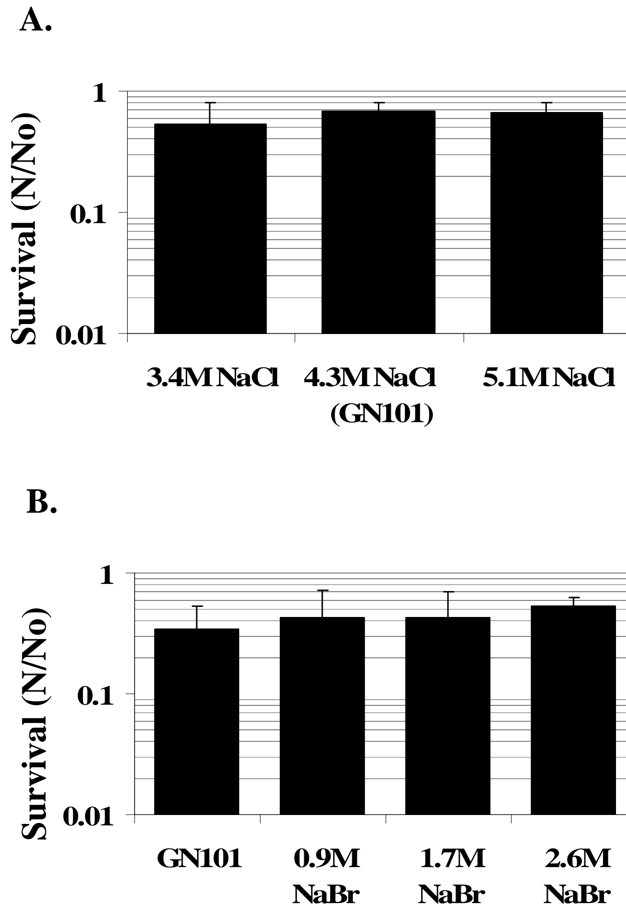


Figure 2-2. *In vivo* survival of *Halobacterium* cells after exposure to 5kGy γ-irradiation. (**A**) *Halobacterium* cells cultured in standard GN101 growth medium (4.3M NaCl), low salt (3.4M NaCl) and high salt (5.1M NaCl) growth media. (**B**) *Halobacterium* cells cultured in standard GN101 growth medium (4.3M NaCl) and in GN101 growth media with molar replacement of NaCl with NaBr as indicated (eg. 0.9M NaBr indicates that of the 4.3M salts in the GN101 growth medium, 0.9M NaCl was replaced with NaBr resulting in a medium composed of 3.4M NaCl and 0.9M NaBr). All data shown is the average of at least 2 replicates with standard errors shown. None of the differences in survival between cells grown in standard GN101 medium and the altered salt conditions were not statistically significant ($P>0.05$).

Chromosomal Fragmentation after Exposure to Ionizing Radiation

DNA double strand breaks (DBSs) have been shown to be a consequence of exposure to ionizing radiation (DiRuggiero et al. 1997; Daly et al. 1994). Here, we measured the effects of intracellular salts concentration on chromosome fragmentation by γ-irradiation in cultures of *Halobacterium* grown in standard GN101 medium (4.3M NaCl) and in bromide medium (GN101 medium with 1.7M NaCl replaced with 1.7M NaBr). Cultures of *Halobacterium* and of the highly radiation-resistant *D. radiodurans* were irradiated at doses from 2.5 to 7.5kGy and DNA fragmentation was analyzed by pulsed field electrophoresis (PFGE). DNA DSBs were observed for all cultures at 2.5kGy and fragmentation increased with radiation doses as visualized by the disappearance of restriction digest DNA bands - seen in the un-irradiated lanes (0Gy) - and the smaller size-range of the DNA smear on the gels (Figure 2-3). *D. radiodurans* showed the highest level of fragmentation at all γ doses followed by *Halobacterium* grown in 3.4M NaCl. *Halobacterium* cells grown with bromide medium showed the fewest DSBs, demonstrated by the fact that restriction digested DNA bands were still visible at 7.5kGy (Figure 2-3B). These *in vivo* data confirmed the decrease of DNA strand breaks after γ-radiation in the presence of halides and the greater protective effect of bromide salts that we observed in our *in vitro* assays.

DNA Base Oxidation after Exposure to Ionizing Radiation

Oxidized DNA bases Fapy-Ade, Fapy-Gua, and 8-oxo-Gua were quantified using GC/MS (see review in (Dizdaroglu et al. 2002)) in DNA from γ-irradiated *Halobacterium* cells cultured in either standard GN101 medium (4.3M NaCl) or bromide medium (GN101 medium with replacement of 1.7M NaCl with 1.7M NaBr); cells were irradiated at increasing doses of γ-radiation, from 2.5 to 7.5kGy. We observed a positive correlation between the number of DNA lesions and the dose rates for oxidized DNA bases (Figure 2-4), with a higher number of Fapy-Gua lesions (up to 6.6 Fapy-Gua/10^6 DNA bases) than Fapy-Ade (2.2 lesions/10^6 DNA bases) after exposure to 7.5kGy of γ-radiation (Figure 2-4). Significantly fewer DNA base lesions (≤2.5-fold difference) were found in *Halobacterium* cells grown in bromide medium than in cells grown in standard GN101 medium (Figure 2-4). The total number of oxidized bases measured per kGy of γ-irradiation was calculated for *Halobacterium* cells grown in standard GN101 medium (4.3M NaCl) as well as *D. radiodurans* for all γ doses and showed an excess of Fapy-Gua lesions over 8-OH-Gua and Fapy-Ade (Figure 2-4C). Recovery of the cells under optimal conditions following irradiation at 2.5kGy showed that Fapy-Gua and 8-oxo-Gua lesions were repaired within 2hrs whereas the Fapy-Ade lesions required nearly 12hrs for repair (Figure 2-5A-C). DNA samples were also analyzed with PFGE during the recovery period. Figure 2-5D shows the reappearance of some restriction digest DNA bands in the PFGE gel after 4 hours of recovery and the reappearance of all restriction digest DNA bands after 8 hours suggesting that DNA DSBs in *Halobacterium* were repaired within 8hrs after irradiation at 2.5kGy.

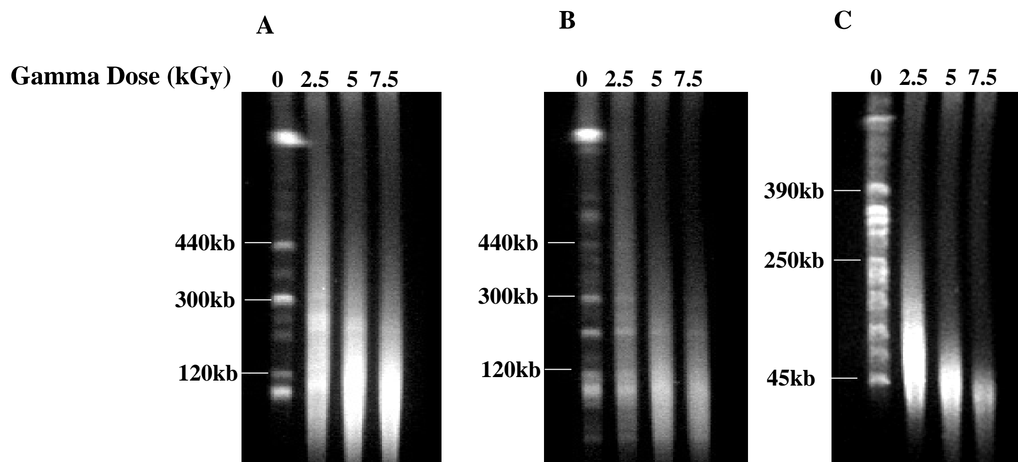


Figure 2-3. PFGE analysis of DNA DSBs in *Halobacterium* and *D. radiodurans* (**A**) *Halobacterium* cells cultured in standard GN101 medium containing 4.3M NaCl. (**B**) *Halobacterium* cells cultured in GN101 medium with 1.7M NaCl replaced with NaBr resulting in a medium composed of 2.6M NaCl and 1.7M NaBr. (**C**) *D. radiodurans* cells cultured in standard TGY medium (no salt). Cell cultures were exposed to 0, 2.5, 5, and 7.5kGy of γ-radiation prior to embedding in InCert agarose plugs at a final density of $1x10^9$cells/mL. The plugs were digested using *Xba*I (*Halobacterium*) or *Not*I (*D. radiodurans*).

Protein Oxidation after Exposure to Ionizing Radiation

Protein oxidation was measured by immunodetection of carbonyl groups in *Halobacterium* protein extracts separated by acrylamide gel electrophoresis and using the OxyBlot Protein Oxidation Detection Kit (Chemicon/Millipore; Billerica, MA). We found that the relative amount of protein oxidation in *Halobacterium* increased with increasing doses of γ-irradiation of cells grown in standard GN101 medium (Figure 2-6A). *Halobacterium* cells cultured in bromide medium showed significantly fewer oxidized carbonyl groups than cells cultured in standard GN101 medium (Figure 2-6B). Analysis of protein cells extracts during recovery of cells grown in standard GN101 medium over a 12-hour period following γ irradiation at 2.5kGy showed a decrease in protein oxidation level over time, and a return to pre-irradiation levels by 8 hours. These results indicate that oxidized proteins in *Halobacterium* were repaired or removed by 8 hours after γ-irradiation (Figure 2-6C).

Halobacterium Intracellular Mn/Fe Ratio

Recent evidence has suggested that manganese may also play a role in prevention of oxidative damage, particularly to proteins (Daly et al. 2004; Daly et al. 2007). Unlike iron, manganese does not participate in Fenton chemistry that produces the highly reactive hydroxyl radical. A high Mn/Fe was proposed by Daly et al. (Daly et al. 2004) as a common marker of radiation resistance in bacteria. The ratio of intracellular Mn/Fe in *Halobacterium* (D_{10}=5kGy) was measured using ICP-MS, and determined to be 0.27, comparable with the reported ratio for *D. radiodurans*

(D_{10}=16kGy) of 0.24, but higher than that of both the less-resistant *Enterococcus faecium* (D_{10}=2kGy) at 0.17 and the radiation sensitive *E. coli* (D_{10}=0.7kGy) with a ratio of 0.0072 (Daly et al. 2004).

Discussion

Exposure to ionizing radiation, like desiccation, results in the production of ROS including the highly reactive hydroxyl radical that can generate oxidative lesions in DNA and proteins. ROS can be scavenged by enzymes such as superoxide dismutase (reviewed in Cannio et al. 2000) or by carotenoid pigments (Saito et al. 1997). Abiotic methods of free radical scavenging such as the scavenging of hydroxyl radical by intracellular chloride have been proposed (Shahmohammadi et al. 1998; Kottemann et al. 2005), and recently data concerning the role of manganese in the scavenging of superoxide radicals has served to highlight the role of the intracellular milieu in radiation resistance (Daly et al. 2004; Daly et al. 2007).

Chloride salts have been linked to DNA protection from heat using NaCl (Masters et al. 1998), KCl and $MgCl_2$ (Marguet and Forterre 1994; Marguet and Forterre 1998). *In vitro* evidence has also linked chloride ions to DNA protection from the effects of ionizing radiation using circular plasmid DNA in 2M KCl exposed to 50Gy of γ-irradiation (Asgarani et al. 1999), and using chromosomal DNA from *Halobacterium salinarium* in 2M KCl and exposed to 200Gy (Shahmohammadi et al. 1998). Here we confirm and extend these findings using 2M and 4M KCl buffered solutions with pUC19 plasmid DNA up to 300Gy of γ-irradiation. It is important to note that 4M

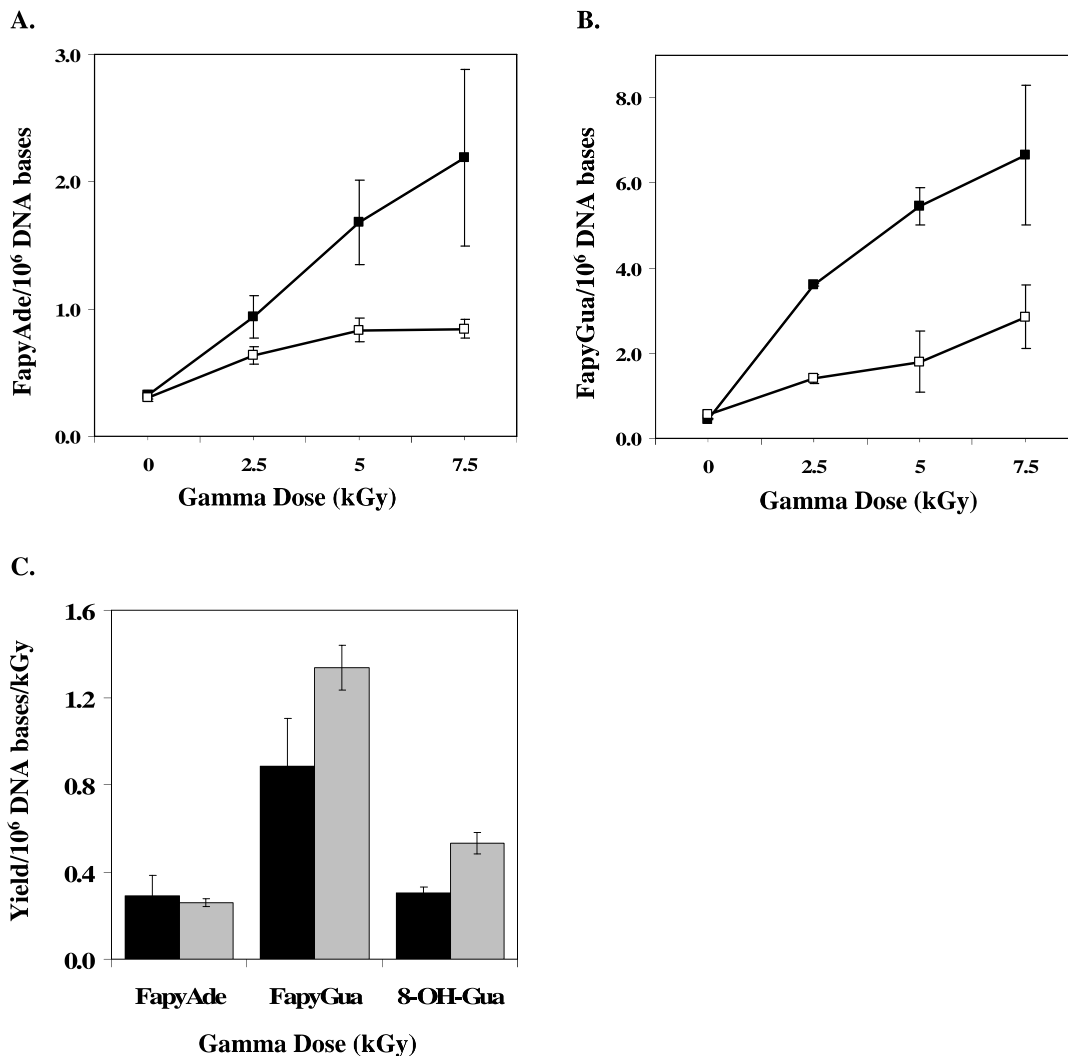


Figure 2-4. DNA base oxidation quantification for *Halobacterium* cells cultured in standard GN101 containing 4.3M NaCl (solid black squares) and *Halobacterium* cells cultured in GN101 with replacement of 1.7M NaCl with NaBr (open black squares). Oxidized base moieties quantified using GC/MS were (**A**) FapyAde and (**B**) FapyGua. Data shown is an average of at least 2 replicates with standard deviations. Yield of oxidized DNA lesions per 10^6 DNA bases per kGy of γ-radiation is shown in (**C**). Black bars represent *Halobacterium* cultured in standard GN101 medium (4.3M NaCl), and light grey bars represent *D. radiodurans*.

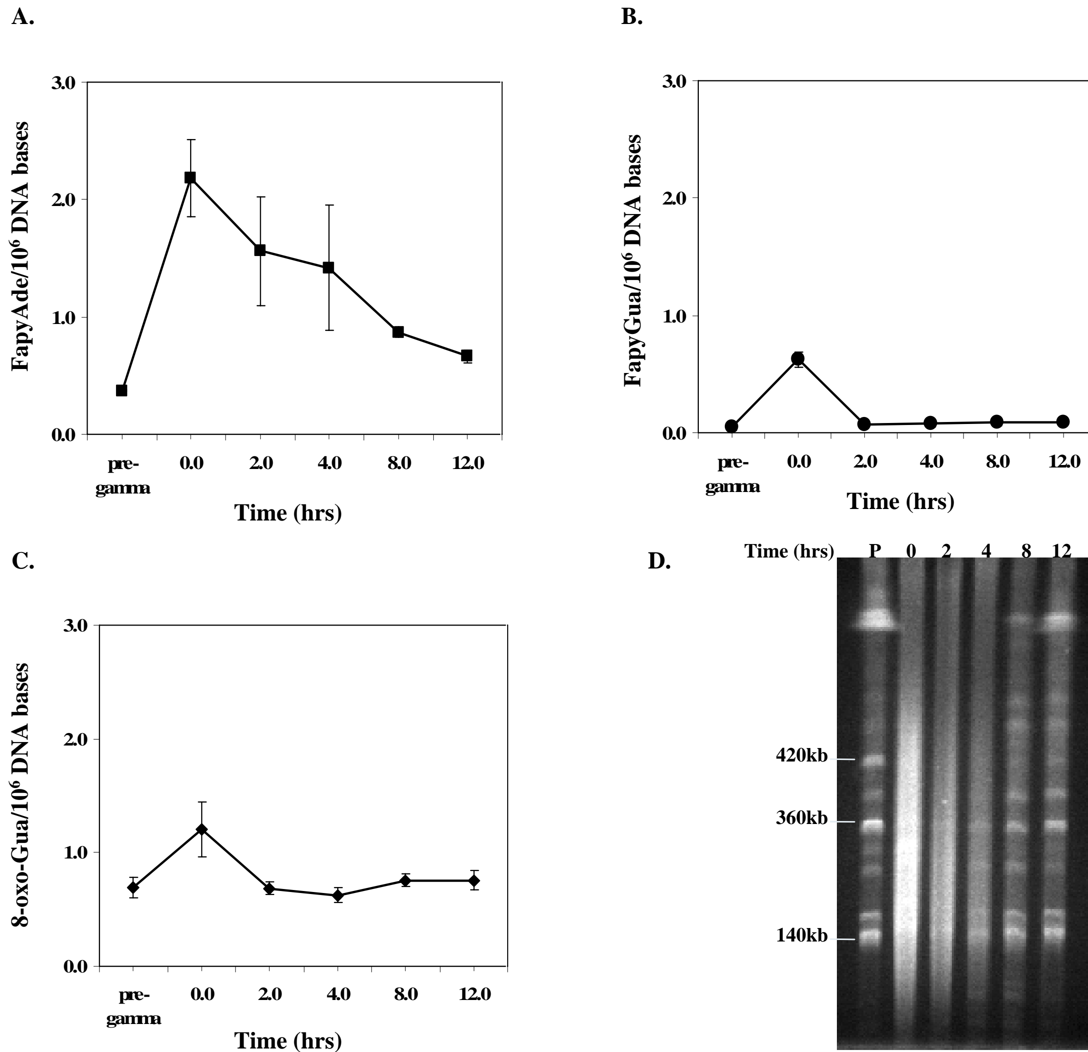


Figure 2-5. Repair of oxidized DNA lesions and DSBs over 12 hours of recovery in *Halobacterium* cells cultured in standard GN101 medium (4.3M NaCl) exposed to 2.5kGy of γ-radiation. Repair of (**A**) Fapy-Ade, (B) Fapy-Gua, and (**C**) 8-oxo-Gua lesions shown as quantified by GC/MS. Data shown is an average of at least 4 replicates with standard deviations. (**D**) PFGE analysis of DNA DSBs repair over time (P=pre-irradiation; 0, 2, 4, 8, and 12 hrs post-irradiation) with agarose plugs containing $1x10^9$cells/mL and digested with *Xba*I.

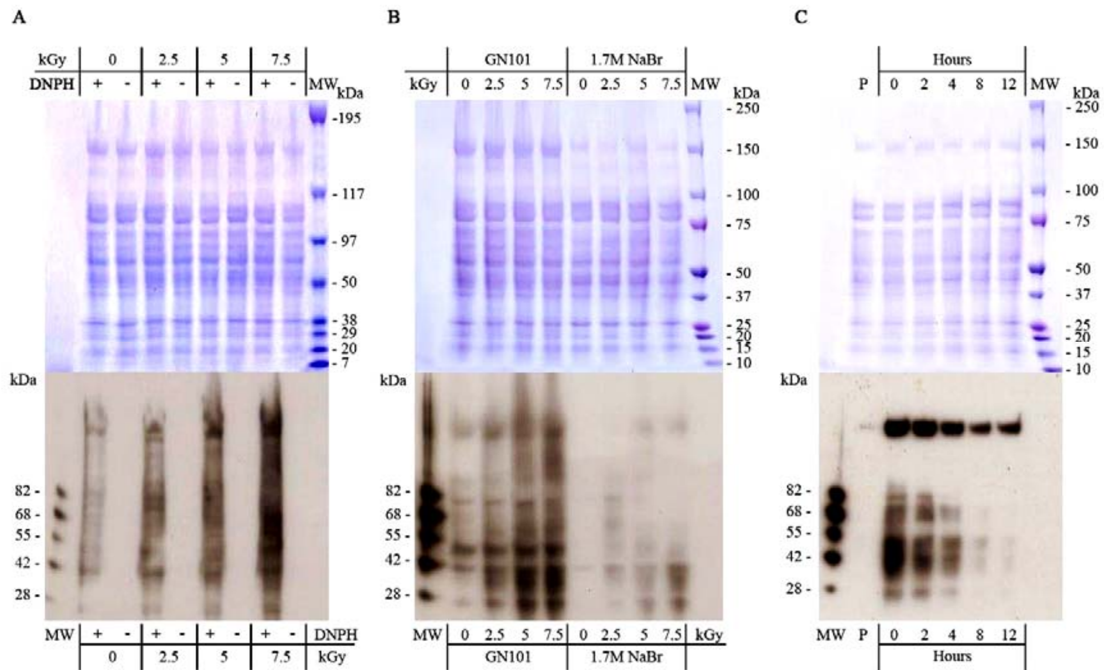
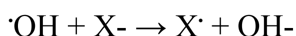


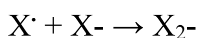
Figure 2-6. Protein oxidation damage in *Halobacterium* after γ-irradiation. Top: Coomassie-stained polyacrylamide gel; Bottom: Western blot immumoassay of protein-bound carbonyl groups. 20 μg of protein were added per lane. Protein extracts were (+) or not (-) treated with DNPH. MW: molecular weight markers. (**A**) *Halobacterium* cells grown in standard GN101 growth medium (4.3M NaCl) were exposed to 0, 2.5, 5, and 7.5kGy of γ-radiation. (**B**) *Halobacterium* cells were grown in GN101 medium (4.3M NaCl) or in GN101 medium with 1.7M replacement of NaCl with NaBr and exposed to 0, 2.5, 5, and 7.5kGy of γ-radiation. (**C**) *Halobacterium* cells grown in GN101 (4.3M NaCl) were exposed to 2.5kGy γ-radiation, followed by recovery at 42°C for up to 12 hours. P, pre-irradiation; 0, 0 hr recovery; 2, 2hr recovery; 4, 4hr recovery; 8, 8hr recovery; 12, 12hr recovery.

KCl is physiologically relevant to *Halobacterium* cells that contain 4M potassium and 4.6M chloride to maintain osmotic balance (Engel and Catchpole 2005). Bromide is present naturally in hypersaline pools, becoming concentrated together with chloride

compounds during periods of evaporation due to the high solubility of halite (Sonnenfeld 1984). For these reasons, 2M and 4M KBr were also tested *in vitro*, and showed a greater protective DNA effect than KCl. Halides (X) have been shown to react with the hydroxyl radical ($^{\cdot}OH$) in a 2-step reaction yielding a halide radical as shown here:



$^{\cdot}OH + X- \rightarrow X^{\cdot} + OH-$



$X^{\cdot} + X- \rightarrow X_2-$

Chloride radicals are less reactive with DNA bases than hydroxyl radicals (Ward and Kuo 1968) resulting in decrease oxidative damage to macromolecules as demonstrated by the protective effect of increase salt concentration in our *in vitro* experiments. The increased DNA protective effect observed in the presence of bromide compared to chloride was likely due to the increased reactivity of bromide ions with hydroxyl radicals. Reported reaction constants (*k*) for Br^- and Cl^- at pH 9 are 10^9 and 10^4 $M^{-1}s^{-1}$ respectively [values taken from Dorfman and Adams (1973); calculated values not available for pH 7].

We used the model halophile *Halobacterium* to test the protective effect of salts against the deleterious effect of γ-generated ROS. Increased survival to γ-irradiation in high salt or bromide medium was not observed using after growth on solid media likely because of the efficient repair capability of *Halobacterium* for DNA damage (Kish and DiRuggiero 2007) and the incubation time required for the cells to form

colonies (up to 10 days). At the molecular level, however, the amounts of DNA DSBs, oxidized bases and oxidized protein residues following γ irradiation were all decreased when 1.7M of NaCl were replaced by NaBr in the culture medium. This is direct evidence of the scavenging role of Br ions for ROS produced by γ-irradiation, resulting in decreased oxidative lesions to the cell's macromolecules. The halorhodopsin protein present in *Halobacterium* cellular membrane was shown to transport both chloride and bromide equally as efficiently into the cell in a light-driven reaction (Steiner et al. 1984) suggesting that the extracellular increase in Br was also reflected in the intracellular environment. The *in vivo* data presented here reflect the *in vitro* observations from our study, and that of others (Shahmohammadi et al. 1998; Asgarani et al. 1999; Saito et al. 1997), demonstrating the physiological relevance of intracellular ionic composition to ionizing radiation resistance.

PFGE analysis of DNA strand breaks showed that DNA from *D. radiodurans* was more fragmented than DNA from *Halobacterium* at the same doses of γ-irradiation, despite the fact that *D. radiodurans* cells are more radiation resistant (D_{10}=16kGy; γ dose at which we observe 10% survival) than that of *Halobacterium* (D_{10}=5kGy). This either suggests an increased dependence on DNA repair mechanisms, such as the extended synthesis-dependent strand annealing pathway followed by recA-dependent homologous recombination proposed for the repair of DSBs in *D. radiodurans* (Zahradka et al. 2006), or a lesser role for DNA DSBs in radiation resistance as suggested by Cox and Battista (2005).

The majority of damage from ionizing radiation results from the formation of ROS in cells (Riley 1994). Therefore, the level of radiation-induced DNA oxidative damage other than strand breakage, and the ability of a cell to repair those damages, should be determined to better evaluate the biological effects of ionizing radiation. A large number of oxidized base- and sugar-derived lesions have been identified in DNA but very few can be accurately measured in cellular DNA (Cadet et al. 2002; Dizdaroglu et al. 2002). For those oxidized products, as little as one-lesion/10^6 DNA bases can be detected by using a combination of enzymatic treatments and LC or GC/MS (Cadet et al. 2002; Dizdaroglu et al. 2002). We quantified three different oxidation products, Fapy-Gua, Fapy-Ade and 8-oxy-Gua in *Halobacterium* DNA following increasing doses of γ-radiation. This is the first study to determine those parameters in a prokaryotic system and the analysis revealed a dose-dependent relationship between the number of DNA lesions and the doses of γ-radiation applied to the cells. Only a few studies on the nature and frequency of DNA base modifications in γ-irradiated cells have been reported and they were exclusively applied to mice, rat or human cell lines (Pouget et al. 2002; Regulus et al. 2007; Frelon et al. 2000). It is therefore difficult to compare the yield of oxidized bases in *Halobacterium* with previous works because of the high doses of ionizing radiation used in our study, 0 to 7500Gy, versus 0 to 450Gy with eukaryotic cells (Pouget et al. 2002; Regulus et al. 2007; Frelon et al. 2000). Nevertheless, Pouget et al. (Pouget et al. 2002) reported the yield of lesions/10^6 DNA bases/Gy for human cells irradiated at 450Gy and found values of 0.005, 0.020 and 0.039 for Fapy-Ade, 8-oxodGuo and Fapy-Gua, respectively. These values are 20- to 50-fold higher, depending on the type of lesions, than what we

observed with *Halobacterium* cells irradiated at 2.5, 5.0 and 7.5kGy, indicating that the damage to DNA is more extensive in the larger eukaryotic cells, probably as a consequence of DNA packaging. It is critical to note that these lesions are indicators for the level of oxidative damage to DNA base and sugar moieties and only represent a small portion of the actual number of DNA decomposition products in the cell (Cadet et al. 2005). If we relate the yield of radiation-induced base damage to cell survival, at the D10 dose (5kGy) *Halobacterium* accumulated 5.4 Fapy-Gua and 1.7 Fapy-Ade per 10^6 DNA bases whereas eukaryotic cells at the D10 dose (5Gy) (Tilly et al. 1999; Wang et al. 2007) accumulated 0.195 Fapy-Gua and 0.02 Fapy-Ade per per 10^6 DNA bases, 20 to 60 less nucleobase lesions. Those results seem to indicate that the repair of oxidative DNA lesions is more efficient in *Halobacterium* allowing these cells to withstand higher levels of damage to their macromolecules. It would be extremely interesting to obtain yields of DNA oxidative lesions, after γ-irradiation, from radiation sensitive strains to find out if this is a common feature among prokaryotes or the result of *Halobacterium* adaptation to desiccation and therefore increased oxidative stress.

The distribution of oxidized DNA bases and nucleosides in eukaryotic systems is not uniform with twice more FapyGua than 8-oxodGuo lesions and 10 times more FapyGua than FapyAde lesions measured after exposure to γ-radiation (Pouget et al. 2002). This is consistent with the idea that guanines are preferential targets for oxidative damage. Guanine residues were found to be sinks for positive charge "holes" (electron holes resulting from photo-ejection of electrons) that are transmitted

from pyrimidines after ionization of DNA by γ-irradiation (Steenken and Jovanovic 1997). The GGG sequence in particular has the ability to draw this positive "hole" through electron hopping motivated by the low ionizing potential of guanine (Giese 2002). This is in agreement with the DNA lesion distribution that we measured in *Halobacterium* and *D. radiodurans*, suggesting that the reactivity of DNA bases with hydroxyl radicals is not altered in presence of chloride, but rather that the conversion of hydroxyl radicals to chloride radicals results in decreased nucleobase damages.

Why Are *Halobacterium* Cells Radiation Resistant?

Models of microbial resistance to ionizing radiation have traditionally focused on DNA repair mechanisms, analyzing the complement and efficiency of repair proteins in radiation resistant species. In this study we present data showing that the intracellular salts found in *Halobacterium*, chloride and bromide species in particular, are responsible for reducing the number of DNA DSBs, oxidized DNA bases and protein residues. Oxidized DNA lesions were repaired in as little as 2hrs post-irradiation while the repair of DNA DSBs occurred within 8 hours post-irradiation, showing that *Halobacterium* possesses effective DNA repair systems for damages that occur despite scavenging of hydroxyl radicals by intracellular halides. Repair of DNA DSBs is aided in both *Halobacterium* and *D. radiodurans* by multiple chromosome copies providing substrates for homologous recombination. *Halobacterium* cells, however, are less radiation resistant than *D. radiodurans* cells despite possessing both intracellular chloride for scavenging DNA-damaging hydroxyl radicals and a high Mn/Fe, which may both reduce hydroxyl radical

production via Fenton chemistry and scavenge protein-damaging superoxide radicals. These findings suggest that DNA DSBs are not the chief source of cyotoxicity after exposure to ionizing radiation. The question becomes which cellular damage from exposure to ionizing radiation is most toxic to cells: DNA oxidation lesions, DNA strand breaks, protein oxidation or possibly lipid peroxidation.

We proposed that it is the combined oxidative damage to all cellular components, including both DNA and proteins, and possibly membrane lipids, which result in cell death after exposure to ionizing radiation. Radiation resistance, thus, is a product of mechanisms for (1) prevention and (2) for repair of oxidative damages to cellular macromolecules. While common elements, such as DNA repair pathways, are intrinsic to most extremophiles, there are likely to be multiple pathways for prevention mechanisms specific to a subset of radiation resistant microorganisms. We have shown here one such mechanism specific to extreme halophiles. The primary function of high concentration of intracellular salts in *Halobacterium* is to provide osmotic balance in hypersaline environments but it also produces increased resistance to ionizing radiation.

Conclusion

We have shown conclusive evidence that intracellular halides including chloride and bromide offer protection to DNA from oxidative damages after exposure to ionizing radiation not only *in vitro*, but also *in vivo*. These findings emphasize the significance of the cellular environment in determining radiation resistance in microorganisms,

showing that molecular adaptations to environmental stresses allow for survival under diverse conditions not necessarily found in natural environments. We also demonstrated that the determination of radiation-induced damage to all the cell's macromolecules is essential for a better understanding of the biological effects of ionizing radiation.

These finding are also relevant to the field of astrobiology with evidence of a former hypersaline pool at Meridiani Planum on Mars (Rieder et al. 2004). Lacking an atmosphere and magnetic shield to reduce the surface solar irradiance, microorganisms on the surface of Mars would be exposed to far greater levels of UV-C (Cockell et al. 2000) and high-energy radiation than are microorganisms on Earth. Our findings show that the salt environment itself may be a protective factor for potential microbial life on the surface of Mars, indicating that high-salt environments showing water modification are excellent areas for surface investigations looking for evidence of life on Mars.

Materials and Methods

Cultures and Growth Conditions

Halobacterium sp. strain NRC-1 cultures were grown in standard GN101 medium [250g/L NaCl, 20g/L MgSO4-7H20, 2g/L KCl, 3g/L Na citrate, 10g/L Oxoid peptone (pH 7.2) with the addition of 1mL/L Trace Elements Solution (31.5mg/L $FeSO_4.7H_2O$, 4.4mg/L $ZnSO_4.7H_2O$, 3.3mg/L $MnSO_4.H_2O$, 0.1mg/L $CuSO_4.5H_2O$)] (Hackett and DasSarma 1989). When specified, the NaCl concentration was changed

from 4.3M to 3.4M or 5.1M, or molar amounts of NaCl were replaced by 0.9, 1.7 or 2.6M of NaBr. All *Halobacterium* cultures were grown at 42°C, 220rpm (Gyromax 737, Amerex Instruments; Lafyatte, CA) to mid-log phase (OD_{600nm}=0.6-0.8). *D. radiodurans* overnight cultures were grown in 25mL TGY medium (1% tryptone, 0.1% glucose, 0.5% yeast extract) (Liu et al. 2003)at 32°C, 220rpm (Innova 4080, New Brunswick Scientific; Edison, NJ), diluted 1:10 the next day and grown to mid-log phase (OD_{600nm}=0.6-0.8).

In vitro Plasmid γ-Irradiation

pUC19 plasmid DNA (2μg) in 1mM potassium phosphate buffered solution (pH 7.2), supplemented by 2M or 4M KCl or KBr, was γ-irradiated to a final dose of 0, 100, 200, and 300Gy using a ^{60}Co gamma source (University of Maryland College Park Gamma Test Facility; dose rate: 3.6kGy/hour). Plamid DNA in 1mM potassium phosphate buffer only was use as control. Samples were then dialyzed against a 1mM potassium buffer (pH 7.2) solution. The extent of DNA single strand break induced by γ-irradiation of the samples was determined by comparing the amount of open circular form plasmid DNA to undamaged supercoiled form plasmid DNA using agarose gel electrophoresis (0.8% TAE, 85V, 75min). The BioRad Molecular Imager Gel Doc XR System (Hercules, CA) was used to quantify the relative intensities of each band and a ratio of open circular form:supercoiled form plasmid DNA was calculated for each salt condition at each dose of γ irradiation.

In vivo γ-Irradiation

Cultures were irradiated to a final dose of 0, 2.5, 5, or 7.5kGy using a ^{60}Co gamma source (University of Maryland College Park Gamma Test Facility, College Park, MD, dose rate = 3-13kGy/hr, and Uniformed Services University of the Health Sciences, Bethesda, MD, dose rate = 4.7kGy/hr). All samples regardless of the volume of the starting culture were irradiated in a volume of 1mL after concentration by centrifugation (8000 x g, 5min) and resuspension in 1mL of the appropriate growth medium in a1.5mL microcentrifuge tube.

Survival assays were performed using 1mL of log phase culture (OD=0.8), which was diluted in BSS (basal salt solution; GN101 medium lacking Oxoid peptone) after irradiation prior to plating.

Aliquots (25mL each) from one of four replicate starting cultures each for *Halobacterium* cells grown in standard GN101 medium, *Halobacterium* cells grown in bromide medium (1.7M replacement of NaCl with NaBr), and *D. radiodurans* cells cultured in TGY medium, were used for GC/MS, Oxyblot, and PFGE analyses. Aliquots were concentrated by centrifugation (8000 x g, 5min) to a final volume of 1mL each in the appropriate medium and transferred to 1.5mL microcentrifuge tubes for irradiation. *D. radiodurans* samples were kept on ice during the irradiation. Following irradiation, the cells were collected by centrifugation (8000 x g, 5min), and the cell pellets flash frozen in a dry ice-ethanol bath for storage at -80°C prior to

sample processing with the exception of PFGE samples that were kept on wet ice after centrifugation and removal of the medium.

Pulsed Field Gel Electrophoresis

0.8% InCert agarose (Lonza; Rockland, ME) plugs were prepared for *D. radiodurans* and *Halobacterium* containing approximately $1x10^9$ cells/mL. *Halobacterium* cell pellets were resuspended in room temperature BSS (250 g/L NaCl, 20 g/L $MgSO_4$, 2 g/L KCl, 3 g/L sodium citrate), mixed with InCert Agarose prepared in 3:1 BSS:dIH_2O rather than TE as was used for the bacterial samples and pipetted into the plug mold (BioRad). Plugs were lysed in proteinase K solution (0.25M EDTA; pH8) (Invitrogen; Carlsbad, CA), 1% N-lauryl sarkosine, and 0.5mg/mL proteinase K at 54°C in air incubator for 1-2 days. Plug washes consisted of 2x1hr in 20mL 1x TE buffer at room temperature, 2x1hr in 20mL 0.5x TE buffer at room temperature, and 4x24hr in 0.5x TE buffer at 4°C to desalt the plugs.

D. radiodurans samples were processed according to the protocol by Harris et al (Harris et al. 2004) with the following changes: cells were collected by centrifugation at room temperature (8000 x g, 5min). ESP buffer contained 0.5M EDTA at pH8 rather than pH 9-9.5. Plug washes consisted of 2x1hr in 20mL 1x TE buffer, 2x1hr in 20mL 0.5x TE buffer.

For all plugs, proteinase K was inactivated using 1.5-2.5mM Pefabloc (Roche; Indianapolis, IN) at 37°C for 2h followed by washing for 1h at 4°C in 20mL 2mM

Tris-HCl pH 8.0, 5mM EDTA pH 8.0 (x3). Plugs were stored in 5 mL of fresh 2mM Tris-HCl pH 8.0, 5mM EDTA pH 8.0, 4°C after wash steps. Plug were digested by incubating for 20min in 250µl 1x restriction enzyme buffer at 4°C followed by 16hr incubation at 37°C in 250µl fresh 1x restriction enzyme buffer with 50U restriction enzyme (XbaI for *Halobacterium* plugs, NotI for *D. radiodurans* plugs (both New England Biolabs; Ipswich, MA). Digested plugs were equilibrated in 1 mL of 2mM Tris-HCl pH 8.0, 5mM EDTA pH 8.0 for 20min prior to electrophoresis. Genomic DNA was analyzed using a CHEF DR-III electrophoresis system (BioRad; Hercules, CA) using 1% PFGE certified agarose (BioRad; Hercules, CA) gels. *Halobacterium* samples were run in 0.25x TBE buffer for 22hrs at 12°C, 6V/cm, 10-60s switching times, 120° included angle. *D. radiodurans* samples were run in 0.5x TBE buffer for 22hrs at 12°C, 6V/cm, 10-60s switching times, 120° included angle. DNA was visualized using ethidium bromide stain.

Genomic DNA Extractions and GC/MS Analysis

Cell pellets from *Halobacterium* were resuspended in 1mL BSS at room temperature and transferred to 250mL centrifuge bottles. Proteinase K (0.13mg/mL) (Invitrogen; Carlsbad, CA) and 2mM desferal (Deferoxamine; Sigma; St. Louis, MO) were added to 75mL of dH_20 (3x original culture volume) to lyse cells at 37°C for 90 minutes. Desferal was used to reduce DNA oxidation during extraction(Helbock et al. 1998). DNA was ethanol precipitated (Sambrook et al. 1989) and the resulting DNA pellets were stored under 70% ethanol at -20°C for later analysis. *D. radiodurans* cell pellets were first washed in 5mL 100% ethanol to strip the outer cell membrane. The cells

were then resuspended in lysis buffer [0.2mg/mL chicken egg white lysozyme (Sigma; St. Louis, MO) and 2mM desferal in 2.5mL TE (10mM Tris 0.1mM EDTA pH8.0)] and incubated in a 37°C water bath for 1hr, followed by addition of 0.2mg/mL proteinase K (Invitrogen; Carlsbad, CA) and 2% SDS and another water bath incubation at 60°C for 4hrs. DNA was isolated by phenol-chloroform extraction (Sambrook et al. 1989) using Phase Lock Gel Light 15mL tubes (5-Prime; Gaithersburg, MD) to aid in phase separation according to the manufacturer's protocol. DNA was precipitated using 2 volumes 100% ice cold ethanol and 1/10 volume 3M sodium acetate (pH 5.2). DNA pellets were stored under 70% ethanol at -20°C prior to analysis.

Protein Oxidation Analysis

25mL culture cell pellets were resuspended on ice in 1mL cold 1M salt buffer (50mM potassium phosphate pH 7.0, 1M NaCl, 1% 2-mercaptoethanol) and sonicated for 30s (Virsonic 100, Virtis, Gardiner, NY) followed by incubation on ice for 30s, repeating 3 times. The cell lysate was then fractionated by centrifugation (8000 x g, 30min, 4°C) retaining the supernatant containing soluble proteins on ice and storing at -20°C. Protein concentration was determined using BioRad Bradford Assay (Hercules, CA) using the manufacturer's protocol. Protein oxidation was detected using the Oxyblot Protein Oxidation Detection Kit (Chemicon/Millipore; Billerica, MA) following the manufacturer's protocol. Briefly, 20µg of protein sample was derivatized and then applied to a 5-20% acrylamide gradient gel (PAGEGel; San Diego, CA) for separation by electrophoresis at 150V, 50 mAmps, for 2.5 hours. The proteins were

then transferred to a PVDF membrane (Millipore; Billerica, MA) via Western transfer at 25V, 300mAmps, for 1.5hours. Immunodetection was performed using 1° and 2° 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 0.5-4min.

Cell Interior Elemental Analysis

A 500mL culture of *Halobacterium* cells was grown in standard GN101 medium to mid log phase (OD_{600nm}=0.6) and collected by centrifugation (8000 x g, 5 min, 4^{0}C). Cells were resuspended in 10mL 1xHigh Salt PBS (pH7.4) (8.24mM Na_2HPO_4, 1.58mM NaH_2PO_4 , 4.4M NaCl), washed twice in 1xHigh Salt PBS and twice in 1xHigh Salt PBS with 1mM EDTA (pH7.4) to a final volume of 30mL. Cells were collected by centrifugation (8000 x g, 5 min, 4^{0}C) and flash frozen in a dry ice-ethanol bath prior to storage at –80°C until analysis. Samples were analyzed at the Pacific Northwest National Laboratory (PNNL) using ICP-MS as previously described (Daly et al. 2004; Posey and Gherardini 2000; Ma et al. 1999).

Chapter 3: Molecular-Level Response of *Halobacterium* sp. str. NRC-1 to Oxidative Damage after Exposure to Ionizing Radiation

Introduction

Organisms of the phylogenetic domain Archaea are environmentally ubiquitous and typically represent ~10% of the microbiota (Robertson et al. 2005). However, in environments characterized by extreme conditions, such as high temperature or salinity, archaea dominate the microbial population due to their unique physiology (Woese et al. 1990). Halophilic archaea, for example, possess a range of mechanisms (Baliga et al. 2004; Kottemann et al. 2005) to endure high levels of solar radiation, greater than 4.0 M salinity, and wide temperature fluctuations, all of which contribute to intermittent desiccation/rehydration cycles. In a previous study we showed remarkably high resistance to one factor, ultraviolet (UV) radiation, and the coordinated genome-wide response involved in survival (Baliga et al. 2004). The physiological robustness of halophiles is further evident in the extraordinary resistance of the model halophile *Halobacterium salinarum strain NRC-1* (heretofore *Halobacterium NRC-1*) to both desiccation and gamma (γ) radiation (Kottemann et al. 2005). Both these challenges induce severe DNA damage including structural modification to nucleotide bases and DNA strand breaks (Hutchinson 1985; Dianov et al. 2001). In case of γ radiation most of the damage results from production of hydroxyl radicals via radiolysis of water (Riley 1994). Therefore, mechanisms to

minimize and reverse oxidative stress are also crucial components of radiation resistance.

Systems approaches enable the elucidation of global physiological responses to environmental perturbations along with underlying regulatory circuits that modulate and coordinate various cellular repair and recovery processes (Kaur et al. 2006). Ideally, a systems approach constitutes the simultaneous analyses of dynamic changes at all levels of biological information processing from DNA, RNA and protein through phenotypic responses. Due to technological and cost limitations, extensive time-series proteomic studies have thus far been limited and hence microarray studies are often conducted with the assumption that transcript level changes sufficiently approximate downstream physiological responses. However, several studies have indicated an uncertain correlation between mRNA levels and downstream proteomic changes (Ideker et al. 2000; Baliga et al. 2002).

We have attempted to address this issue through a systems level time-course study of *Halobacterium NRC-1* response to γ radiation using whole-genome microarray analyses of mRNA transcript levels and quantitative mass spectrometry analyses of total proteins. Through these analyses we gained an overview of the regulatory and functional aspects of cell recovery after γ irradiation. Our finding of a high level of correlation (Pearson coefficients > 0.5) between mRNA and protein levels upon including an appropriate time lag demonstrates that transcriptional changes do indeed translate into protein level changes in a physiologically meaningful manner.

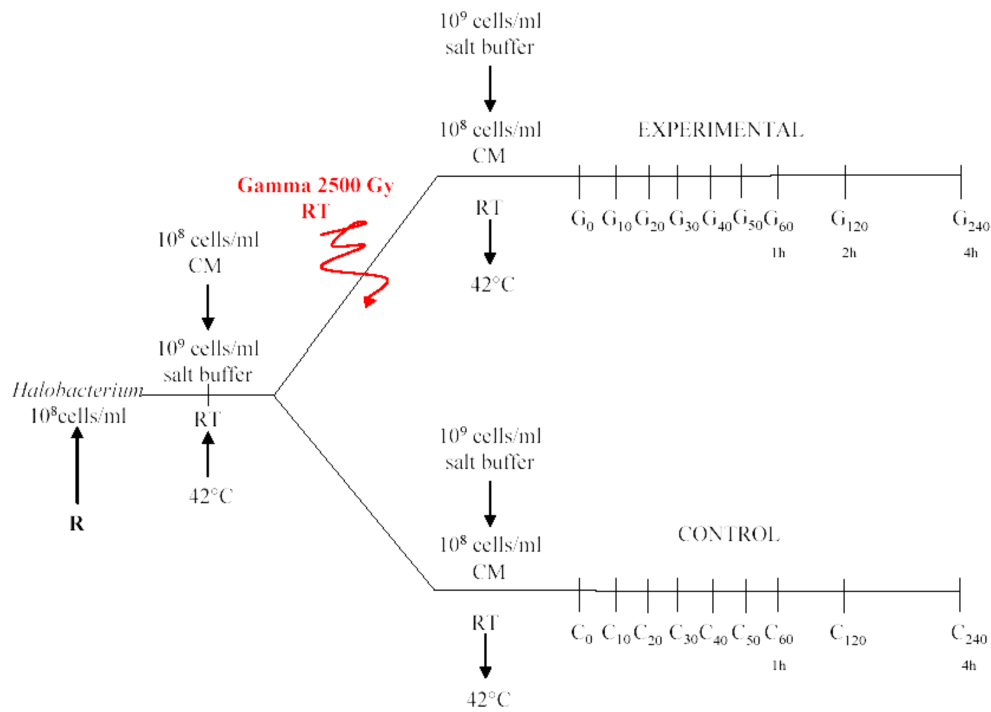
Results

The physiological response of *Halobacterium NRC-1* during recovery from irradiation with 2.5kGy of ^{60}Co γ-radiation was examined temporally at mRNA and protein levels. Unintended perturbations were discounted with identically processed but unirradiated cells (see Figure 3-1 for experiment design). While mRNA level changes were measured over the entire time course (240 min), protein abundance changes were measured at 30, 40 and 60 min in both irradiated and control cells.

mRNA Level Changes

(This analysis was done jointly by the author and Kenia Whitehead at the Institute for Systems Biology after sample irradiation and RNA extraction by the author)

Significance of change in mRNA levels in microarray data was estimated with a likelihood ratio test (Ideker et al. 2000). Comparison of identically processed biological replicates yields maximum likelihood statistic lambda (λ) values consistently below 15 for over 99% of all genes. Therefore, changes discussed heretofore are associated with λ >15.0 and correlate to >99% confidence level. Based on these statistical parameters, 216 genes (~9% of all predicted genes) were differentially expressed of which 143 were upregulated and 73 were downregulated (Figure 3-2).



R: reference RNA; C: control; G: experimental

Figure 3-1. Experimental design for the global analysis of gamma response in *Halobacterium* NRC-1. Cells were harvested and concentrated via centrifugation before gamma exposure. After γ irradiation at 2.5kGy, cells were diluted back to their original volume in CM. Time points were taken as the cultures recovered. Both the control and irradiated samples were treated in the same manner. RT: room temperature.

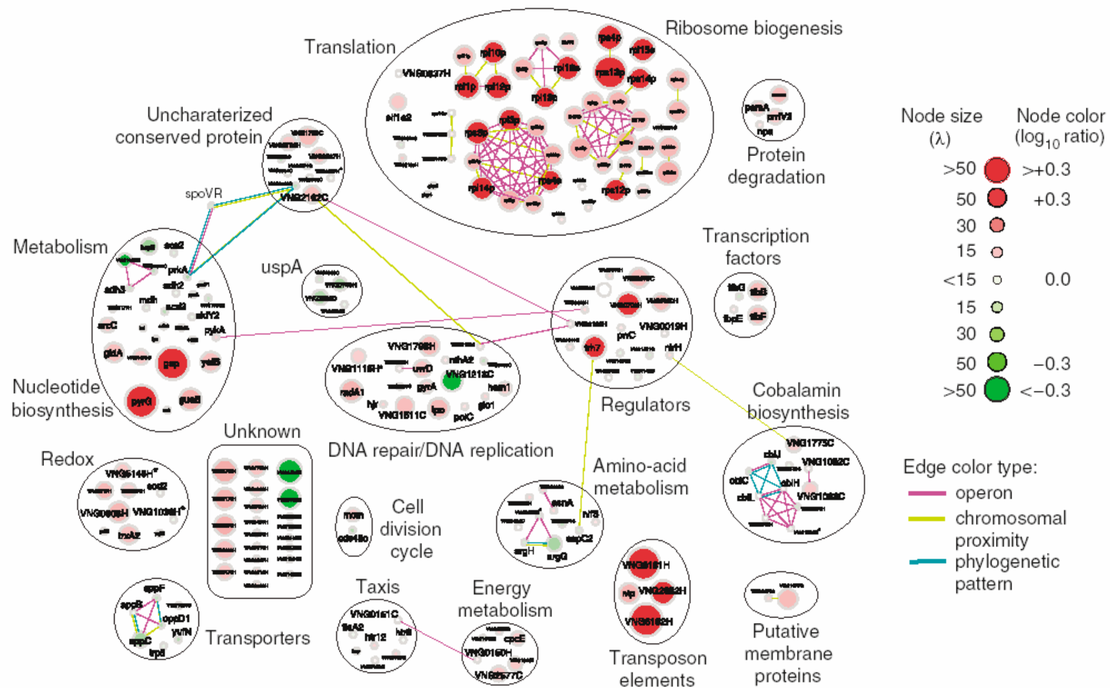


Figure 3-2. Systems level visualization during early response to γ radiation for all genes showing systematic and significant expression changes with the layout organized by general function. The RNA changes are visualized as a network of genes (nodes) and their interactions (edges). The color of the nodes indicates upregulation (red) or downregulation (green) of that gene, whereas the size of the node relates to the significance of the observed change (λ value; see legend). Edge types are color coded to delineate the association type (see legend).

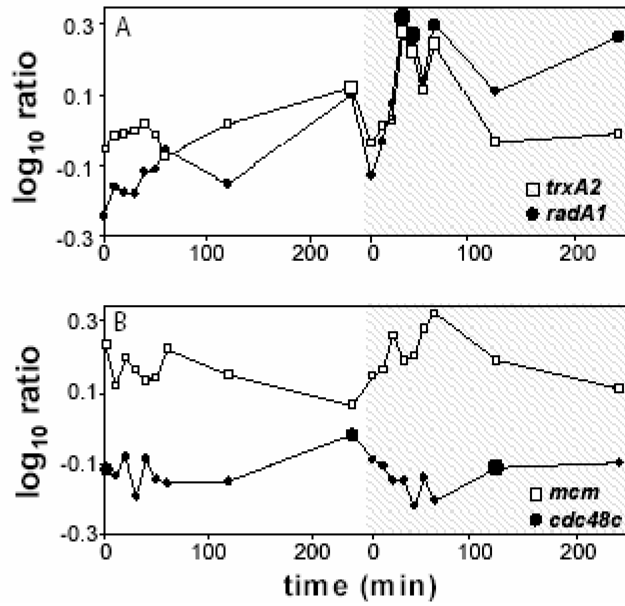


Figure 3-3. Expression profiles ($\log_{10}$ ratios) for *radA1*, *trxA2*, *mcm* and *cdc48C* from one of the two replicate experiments of control and gamma irradiated (shaded area) cultures over time (min). The size of the symbol relates to the statistical significance of the change (λ).

Protein Level Changes

(This analysis was done by Kenia Whitehead at the Institute for Systems Biology)

We measured proteomic changes using quantitative tandem mass spectrometry analyses of four-plexed combinations of trypsinized total proteins labeled with amine-reactive isobaric iTRAQ reagents. The iTRAQ chemistry was expected to yield significantly better proteome coverage relative to the ICAT approach considering that primary amines are more abundant in proteins than the cysteines targeted by ICAT. Indeed, the 1033 proteins detected using iTRAQ represented over three-fold better coverage relative to ~300 proteins identified in a comparable ICAT analysis of *Halobacterium NRC-1* (Baliga et al. 2002). Protein products for 99 of the 143 γ irradiation-induced transcripts were detected, of which 68 had significant abundance changes relative to the control (Figure 3-3 and Supplementary Table 3-2 in (Whitehead et al. 2006)). Likewise, products for 44 proteins were detected for the 73 downregulated transcripts, and the abundances of 27 were significantly perturbed relative to the control. The lists of mRNAs and proteins that changed significantly are provided in Supplementary Tables 3-1 and 3-2 in (Whitehead et al. 2006).

In the sections below we provide (I) a synthesis of the cellular response based on simultaneous analysis of transcript and protein level changes along with evolutionarily conserved functional associations and protein-DNA interactions and (II) a discussion on comparison of changes at mRNA levels to corresponding changes in protein abundance.

Discussion

A systems model for physiological response to γ–radiation

High energy γ particles cause radiolysis of water, generating reactive oxygen species (ROS) (Hutchinson 1985) resulting in oxidative stress and damage. While the high intracellular concentration of KCl and bacterioruberins in haloarchaea have been hypothesized to provide protection by quenching ROS (Carbonneau et al. 1989), this alone is insufficient to alleviate stress induced from γ irradiation (Kottemann et al. 2005) requiring extensive repair and recovery processes. We have assimilated all transcript (Figure 3-2) and protein level changes during recovery from γ irradiation into a physiological model of *Halobacterium NRC-1* which involves restoration of genome integrity, modulation of dehydrogenases, redoxins and cytochromes to minimize ROS reactions, and inhibition of cell division (Figure 3-4).

Physiological Changes

DNA repair to restore genome integrity appears to be primarily mediated by homologous recombination and glycosylase activity. Archaeal homologous recombination proteins are structurally and functionally similar to eukaryotic counterparts of this process (Allers and Ngo 2003). Of the two RecA/Rad51 homologs in archaea, RadA (also called RadA1) and RadB (also called RadA2), only RadA can catalyze strand exchange (Komori et al. 2000a). In *Halobacterium NRC-1* RadA1 mRNA and protein levels increased during early γ response which parallels

similar DNA damage-responsive regulation of this gene in other organisms (Liu et al. 2003) (Figure 3-5, 3-6). Likewise, the branched structure-specific endonucleases, *hjr* (Holliday junction resolvase), was also up regulated after γ-irradiation.

Communication between DNA replication, repair and cell cycle progression is imperative to maintain genomic stability (Sancar et al. 2004). An inverse relationship was observed between mRNA changes of Mcm (upregulated) and Cdc48c (downregulated), a CdcH ortholog putatively involved in cell division (Figure 3-3, bottom panel) implying a pause in the cell division cycle as has been observed in other organisms (Rieger and Chu 2004; Sancar et al. 2004) putatively to ensure completion of DNA repair prior to cell division. In accordance with a pause in the cell cycle, a transient global downregulation was observed similar to that seen after UV irradiation (Baliga et al. 2004) (Figure 3-7). However, unlike the UV response, key recovery related pathways such as nucleotide biogenesis (PyrG), protein degradation (VNG0557H, PsmA), ribosome biogenesis (43 genes) and DNA repair were upregulated (Figure 3-7).

Upregulation of nucleotide biosynthesis genes including *pyrG* (CTP synthase) and *cmk* (cytidylate kinase) indicates increased *de novo* synthesis of nucleotides, which is consistent with damage responses observed in both higher eukaryotes (Rieger and Chu 2004) and *D. radiodurans* (Liu et al. 2003). Increased nucleotide production may be necessary to accommodate increases in transcription and for DNA replication and repair. In line with this, deletion of *pyrF*, which encodes an enzyme central to

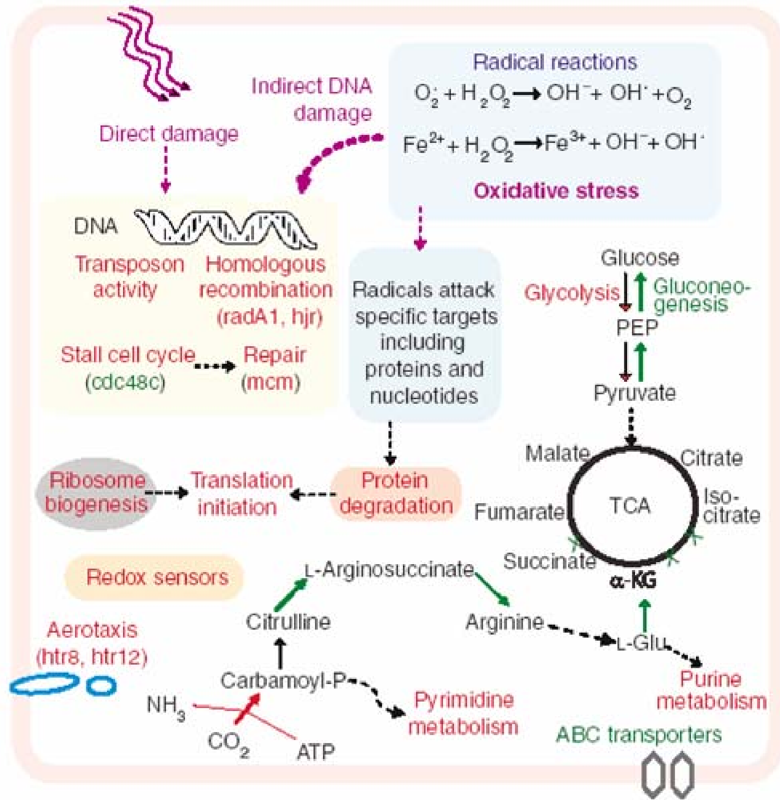


Figure 3-4. Graphical representation of the systems level response in *Halobacterium* NRC-1 to direct and indirect effects of γ-radiation showing both repair and oxidative strategies for cell recovery. Text or arrow color indicates whether the cellular process or pathway was upregulated (red) or downregulated (green). Green X's indicate downregulated steps in the TCA cycle. Blue ellipses represent Htr8 and Htr12.

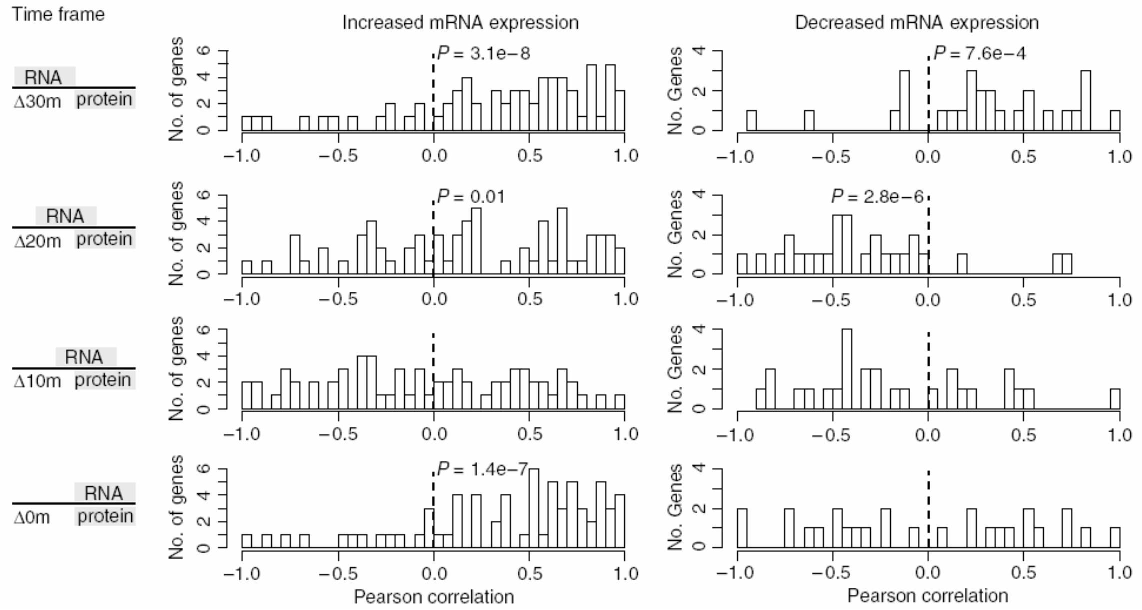


Figure 3-5. Time-lagged Pearson correlations between mRNA and protein abundance for genes with significant changes in both. The time lag is given on the side ranging from 30 min (Δ30m) to no shift (Δ0m). P-values indicate the statistical significance of the distribution of Pearson coefficients and are only stated when a significant skew is observed. P-values on the right of the dashed line indicate a skew towards positive correlations, whereas those on the left indicate a negative correlation.

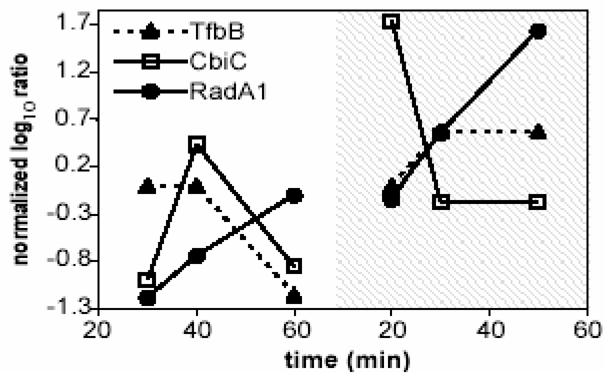


Figure 3-6. Normalized $\log_{10}$ ratios of protein abundance of RadA1, TfbB and CbiC for both the control and γ-irradiated (shaded area) cultures over time (min). The size of the symbol relates to the statistical significance of the change.

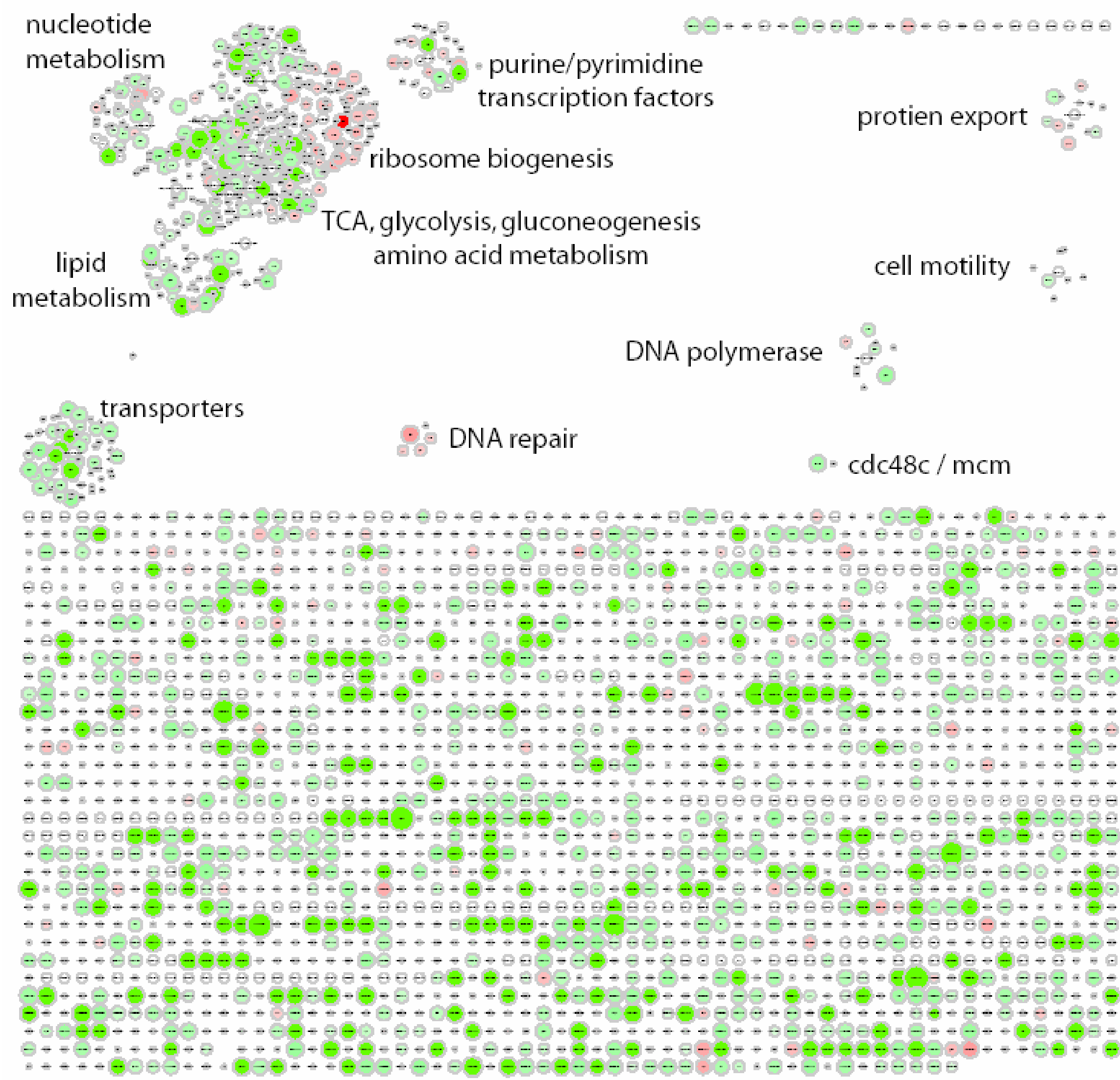


Figure 3-7. Cytoscape view of the *Halobacterium* NRC-1 genome 40 min after γ-irradiation showing global repression with the exception of ribosome biogenesis clusters and DNA repair associated functions (for description of symbols, see legend Figure 3-2).

pyrimidine biosynthesis, significantly reduced resistance of *Halobacterium* NRC-1 to γ–irradiation (see Appendix 1). An increased nucleotide pool would also serve to support enhanced ribosome genesis (43 ribosomal genes were upregulated).

Electron transport systems are especially sensitive to increased ROS production (Imlay 2003). Therefore, the observed downregulation of 8 dehydrogenases (e.g. *adh2*, *adh3*, *sdh* and *mdh*) may reflect depletion of intracellular reducing equivalents during severe oxidative stress (Golden and Ramdath 1987) and an attempt to minimize ROS production by subsequent auto-oxidation reactions (Imlay 2003). Likewise, increased protein abundances for superoxide dismutase Sod2 and redox-related functions such as thioredoxin (*trxA2*; Figure 3-3, top panel) might serve to scavenge free radicals (Cannio et al. 2000). Modulation of general cell metabolism may indirectly stem from these attempts to minimize oxidative stress; for example, as in *D. radiodurans*, several TCA cycle related dehydrogenases in *Halobacterium NRC-1*were also downregulated (Liu et al. 2003; Ghosal et al. 2005).

The simultaneous analysis of transcriptional and translational changes has provided functional relevance for our observations enabling the synthesis of a coherent overview of physiological adjustments necessary for withstanding extreme levels of γ–irradiation. In the next section, we will discuss aspects of transcriptional regulation for coordinating these various processes.

Transcriptional Control

The construction of a regulatory map allows for initial hypothesis formation on the transcription factors and regulators directly mediating a stress response. In *Halobacterium* NRC-1 transcription is mediated by a ~12 subunit eukaryotic-like RNA polymerase II enzyme (RNAP), 6 TATA-binding proteins (TBPs) and 7 Transcription Factor IIB ortholog (TFBs) (Baliga et al. 2000) {Geiduschek, 2005 507 /id}. Transcription is further modulated by approximately 130 additional proteins orthologous to bacterial regulators (Sivaraman et al., 2005). During the γ response at least 9 regulators were upregulated along with one TBP (*tbpE*) and two TFBs (*tfbB* and *tfbF*). Downregulation of 5 regulators was observed, two which were of unknown function and have been newly annotated (Supplemental Table 3-3 in (Whitehead et al. 2006)). Besides the 5 regulators, one TFB, *tfbG*, was also repressed during early stages of the response.

We integrated all significant mRNA changes with a physical map of genome-wide protein–DNA interactions for seven TFBs (Facciotti et al. 2007) to investigate whether some of them may have specialized roles in the γ stress response. Of the 216 γ-responsive genes, transcription factor binding sites could be identified for 39% of them (84 genes) including 12 transcription regulators and kinases (Supplementary Table 4, gray boxes in (Whitehead et al. 2006)). An important point to note is that during the response to γ–radiation, tfbB, tfbF and tfbG were differentially regulated and binding sites for at least one of these are observed in all but 10 of the 84 genes, representing a statistically significant enrichment of these binding sites (P-value =

0.002). In fact, binding sites for TFBb and TFBf, the two TFBs upregulated by γ-irradiation, were the most prevalent (> 1.3-fold enrichment, P-value < 0.009) upstream to genes that were also transcriptionally modulated during the γ response (Supplementary Table 5 in (Whitehead et al. 2006)). In other words, although the presence of other TFB binding sites and possible condition-specific promoter binding by these GTFs complicate inference of exclusive stress-specific control by these TFBs, our data suggest statistically significant association between distribution of TFBb, TFBf and TFBg binding sites and transcriptional modulation of downstream genes in response to γ irradiation. Further, the differential regulation of *tfbB, tfbF* and *tfbG* in response to several stressors (Baliga et al. 2004; Kaur et al. 2006) motivates the hypothesis that these three TFBs coordinate various aspects of physiology that together constitute complex cellular responses during adjustment to diverse stress agents (Facciotti et al. 2007). This hypothesis will be tested in future experiments for a mechanistic understanding of stress response and its regulation.

Comparison of Changes in mRNA Levels and their Corresponding Protein Abundances

The relationship between changes in an mRNA and a corresponding change in its protein abundance is a function of their synthesis and degradation rates as well as their stability. Although there is no evidence for ubiquitin-ligase mediated targeted protein degradation in halophilic archaea, a recent study has demonstrated likely involvement of C-terminal degradation signals for proteasomes in protein stability

(Reuter and Maupin-Furlow 2004). Furthermore, transient up or downregulation of some genes (Kaur et al. 2006), differing rates of protein synthesis and post-transcriptional protein modifications, all lend further complexity in correlating transcription and translation.

We have addressed the sequential nature and therefore likely temporal separation of these processes by calculating Pearson correlations (PC) for each gene by temporally shifting the protein level changes with respect to mRNA level changes; for example, protein levels at 30, 40 and 60 min compared with mRNA levels at 10, 20 and 40 min represents a time lag of 20 min (Δ20m). This analysis was conducted for all 95 genes with significant changes in both mRNA and protein abundances. The resulting correlations for each gene were binned (bin size = 0.05) and are presented as histograms with p-values indicating the significance of the distribution of correlation values (Figure 3-5).

A high degree of correlation was observed for upregulated genes and their protein abundance at each time lag interval except at Δ10 min (Figure 3-5). Transcript and protein levels for DNA repair genes (e.g. RadA1 and UvrD) had highest correlations (PC>0.6) over virtually all time lags perhaps due to a continuous increase in both over the entire time series. Further, while ribosomal proteins encoded within the two major operons had highly correlated changes in transcript and protein, those encoded by genes elsewhere in the genome were less correlated. Likewise, while TfbB and TfbF mRNA and protein level changes were highly correlated to one another in

absence of any time lag, TbpE protein abundance change was manifested after a 30 minute time lag (Figure 3-5). In fact, transcript and protein changes for most down regulated genes were significantly correlated only after a time lag of 30 min. Thus, our analysis confirms that transcript and protein levels changes vary gene-by-gene and on a temporal scale, and further that simplistic global correlations of mRNA and protein level changes at steady state might sometimes be misleading.

Conclusion

In this study we have identified the cooperative physiological mechanisms that render *Halobacterium* NRC-1 resistant to γ radiation and have shown that these are reflected at both the transcript and protein level. This study further supports the view that transcript level changes might indeed be truly reflective of a significant fraction of protein level changes and therefore the physiological manifestations of those changes. Further, as general discordance between steady state mRNA and protein levels are generally attributed to post-transcriptional regulation (Ideker et al. 2001; Gygi et al. 1999), our observation of relatively high degree of correlation between temporally shifted mRNA and protein levels demonstrates the potential importance of the time dimension while interpreting mechanisms of genetic information processing.

Methods and Materials

Strains and Culturing

The wild type strain of *Halobacterium salinarium strain* NRC-1 was used for the gamma radiation experiments (Ng et al. 2000) and the Δ*ura3* mutant strain was from Dr. M.P. Krebs (Illinois State University, Normal, Illinois). Culturing of all strains was done in a liquid Complete Medium (CM; (2); at 42ºC with shaking at 220 rpm (Innova 4080, NewBrunswick Scientific, Edison, NJ).

Gamma Radiation and Response Time Course

γ-irradiation experiments were conducted as follows: Cell pellets from two 180mL cultures (control and experimental) of *Halobacterium* NRC-1 (OD600nm= 0.4) were resuspended in 1/20 volume in a basal salt solution (CM without peptone) and exposed to 2.5kGy of γ-radiation at 22°C (measured with an Omega Engineering Model HH 611PL4F Type K logging thermometer during irradiation) using a 26,000-curie (9.6E14 Bq) ^{60}Co gamma source at Univ. of Maryland College Park Gamma Test Facility at a dose rate of 62.01 Gy/min. Irradiated and control cultures were resuspended in the original volume of CM, split into 20mL aliquots in baffled flasks and incubated at 42°C and 220rpm shaking. Time course samples were placed on ice, pelleted (5000 x *g*, 4°C, 5min) (Figure 3-1) and flash-frozen in a dry ice/ethanol bath after decanting the supernatant. RNA extractions were performed using the Stratagene Absolute RNA kit (La Jolla, CA) and RNA quality checked with the Agilent Bioanalyzer (Palo Alto, CA) and with PCR.

Microarrays

Microarrays were fabricated at the Institute for Systems Biology Microarray Facility. The arrays contain 4 spots per unique 70-mer oligonucleotides for each of 2400 non-redundant genes in *Halobacterium* NRC-1. Labeling, hybridization and washing have been previously described (Baliga et al. 2002). Bias in dye incorporation was accounted for by reversing the labeling dyes (dye-flip). Raw data was processed and converted into log10 ratios with lambda (λ) values determined by the maximum likelihood method (Ideker et al. 2000).

Quantitative Proteomics: iTRAQ Reagent Labeling, μLC-MS/MS and Data Analysis

Proteomic analysis was conducted at three time points (30, 40 and 60 min) for both control and γ irradiated cultures. Relative quantitation was achieved using shotgun isobaric tagging with iTRAQ reagents (Applied Biosystems, Foster City, CA) (Choe et al. 2005; Zhang et al. 2004). The primary amine-specific iTRAQ reagent tags virtually all proteins/peptides except those lacking both lysine and reactive N-terminal amino acids. Quantitation is achieved upon tandem MS, which fragments the iTRAQ reagents unevenly to release daughter products of differing mass (m/z 114, 115, 116 and 117). For direct comparison across multiple runs a common reference sample derivatized with the 114 mass tag was included in each fourplex experiment. Soluble proteins were recovered by centrifugation of cell lysate (prepared by

resuspending the cell pellet in 1mL of water + 1mM of PMSF (protease inhibitor)) and the insoluble proteins were dissolved in 3mL of 10% SDS and centrifuged again. The combined (soluble + insoluble) protein preparation was treated with Benzoase nuclease (25U/μl, 37°C, 45 min) to remove nucleic acids and acetone precipitated. iTRAQ labeling was conducted as per manufacturer's instructions (Applied Biosystems). In brief, in each experiment 4 different protein samples (100 μg each) were separately denatured and reduced with tributylphosphine (TBP; 60°C, 1hr), blocked with Cysteine Blocking reagent, trypsinized (37°C, 1hr), labeled with one of four isobaric reagents (114,115, 116 and 117) (25°C, 10min) and mixed in equimolar ratios. The combined preparation was desalted by cation exchange, diluted with Buffer A (1:1) and acidified (4.5% of H_2PO_4 to pH = 2.9), fractionated by HPLC (Integral 100 Q, Applied Biosystems) and further desalted using UltraMicroSpin columns (Nest Group, Southborough, MA). The fractions were dried, dissolved in 0.4% acetic acid and analyzed by μLC/MS/MS analysis on an Applied Biosystems API QSTAR Pulsar I mass spectrometer, equipped with an in-house nanospray device using standard procedures.

Peptide and protein identification was achieved with COMET, SEQUEST, PeptideProphet and ProteinProphet and relative quantitation was conducted using the Libra algorithm (Keller et al. 2002; Nesvizhskii et al. 2003; Pedrioli et al. 2004). Libra integrates signal intensities of the reagent mass/charge (m/z), normalizes each peptide for a given protein by the sum of its channel intensities (114, 115, 116 and 117 isobars), removes values more than 2 standard deviations (σ) from the mean,

recalculates the mean, and lastly calculates the 1- σ errors to improve the consistency of quantitation (Choe et al. 2005). The data for the two iTRAQ sets were then merged and loaded into the Gaggle suite (Shannon et al. 2006).

Protein abundance changes were observed for key cellular processes including DNA repair and replication (RadA1, UvrD and GyrA), ribosome biogenesis (35 proteins detected), protease activity (PsmA1 and VNG0557H), transcriptional regulation (TfbB, TfbF, TfbG, Boa3, PrrC and Prrlv2), and several enzymatic steps from various biochemical pathways (e.g. ArcC, PyrG, YafB, CbiC) including 6 of the 8 downregulated dehydrogenases. All proteomic changes are consistent with our interpretations of physiological responses to γ radiation solely on the basis of transcriptional analysis. Due to the limited number of samples analyzed (three time points) and paucity of data for some individual proteins we are unable to cluster the protein data in a statistically meaningful manner. We further detected gene products for 4 ORFs not included among the initial gene predictions during the *Halobacterium NRC-1* genome annotation stressing the potential for proteomic analysis to add to the gene enumeration of classical methods for gene prediction. All four new ORFs have pI < 4.5 typical for halobacterial proteins (Goo et al. 2003) and putative functions for two are given in Supplemental Table 3-3 in (Whitehead et al. 2006).

Data Integration and Visualization

Data analysis was performed using the Gaggle (Shannon et al. 2006) and several of its inter-linking components including Cytoscape (Shannon et al. 2003), Data Matrix

Viewer (DMV), a 'kegg wbi' for metabolic pathways and Tile Viewer to examine ChIP/chip data (Facciotti et al. 2007). Statistical analysis was conducted with the TIGR MeV program (Saeed et al. 2003) and the R package (www.r-project.org).

γ-radiation survival of Δ*ura3* strain

Observed gene expression changes could be associated with direct repair of cellular damage, with pathways that manufacture necessary metabolites for the repair and recovery process or with an attempt to energetically accommodate costly repair processes. We tested the hypothesis that increased synthesis of nucleotide biosynthesis genes post-γ irradiation was necessary to provide the nucleotide pool required for DNA repair and replication activity by assaying the phenotype of a Δ*ura3* strain. This strain is deficient in orotidine 5'-phosphate decarboxylase and therefore defective in *de novo* uracil biosynthesis. Cell survival was evaluated in triplicate at 5000 Gy (N/No = 0.11 for wildtype cells) by counting survivor colonies on agar plates (Kottemann et al. 2005; Peck et al. 2000). Average N/No of the Δ*ura3* strain was 0.095 + 0.007 while wild type cells have an average N/No of 0.276 + 0.024. Reduced survival of Δ*ura3* strain (34.4% lower than wildtype) is consistent with the importance of *de novo* nucleotide biosynthesis during recovery from γ-radiation damage.

Chapter 4: The Role of Mre11 and Rad50 in the Repair of DNA Double Strand Break Repair in the Halophilic Archaeon, *Halobacterium* sp. str. NRC-1

Introduction

Maintaining genome integrity through the repair of DNA double strand breaks (DSBs) is critical to cell survival. The repair of those highly cytotoxic lesions is particularly essential following exposure to ionizing radiation, which has been shown to produce extensive DNA fragmentation (DiRuggiero et al. 1997; Daly et al. 1994). Multiple pathways for repair of DNA DSBs have been identified, including synthesis dependant strand annealing (SSA) both with and without extended DNA synthesis (Paques and Haber 1999; Zahradka et al. 2006), non-homologous end joining (NHEJ), and homologous recombination (HR), working in a coordinated manner on the various damage types (Swanson et al. 1999; Slupphaug et al. 2003). Although the pathways utilized depend on the protein complement of the species, the cell cycle, and the number of genome copies present, some elements are shared between pathways. The involvement of a cross-over structure and a recombinase such as RecA (Bacteria), RadA (Archaea) or Rad51 (Eukarya) is common to both HR and extended synthesis dependant strand annealing (ESDSA), and the processing of dsDNA ends to produce a 3' overhang is found in HR, SSA, and ESDSA (Zahradka et al. 2006).

The process of HR repair of DNA DSBs in both Bacteria and Eukarya has been extensively studied (see reviews in (Symington 2002; Krogh and Symington 2004; Wyman et al. 2004; Kowalczykowski et al. 1994; West 2003)). The first step in the HR repair pathway is recognition of the double strand break and resection of 5' strand to produce a 3'-OH overhang that can be recognized by the recombinase. In Bacteria, this is primarily done by the RecBCD complex, although there are redundant pathways pathway for RecBCD activities including the use of the RecFOR complex for recombinase loading along with the RecQ helicase and RecJ nuclease for DNA end processing (Wyman et al. 2004). Bacterial SbcCD is the structural homolog of the Eukaryotic Mre11/Rad50 complex (Kowalczykowski et al. 1994). SbcCD has been shown to cleave hairpin DNA, which can block stalled replication forks, prior to homologous recombination rescue of the replication fork (Connelly et al. 1998). Deletions of *sbcC* together with *sbcB* encoding the ExoI 3'-5' exonuclease, has been shown to complement Δ*recBC* in *Escherichia coli* by shunting repair from the RecBCD pathway into the RecFOR pathway (Kowalczykowski et al. 1994). In *Bacillus subtilis* and *Deinococcus radiodurans*, deletion of *sbcC* results in increased sensitivity of the cells to ionizing radiation (Mascarebhas et al., 2006). Deletions of *sbcC* and/or *sbcD* in *D. radiodurans* also result in delayed repair kinetics of DNA DSBs following γ-irradiation (Bentchikou et al. 2007).

The Mre11-Rad50 complex in the Eukarya performs DSB recognition (see reviews in (Dudas and Chovanec 2004; Symington 2002; Assenmacher and Hopfner 2004; van den et al. 2003)). Rad50 has ATP-dependant DNA binding activity provided by

Walker A and B motifs at the termini of the protein. Coiled-coil domains are separated by a zinc hook found to be required for the repair of DSBs by HR (Wiltzius et al. 2005) (see Figure 4-1). Mre11 is a nuclease with both dsDNA exonuclease and ssDNA endonuclease activities as well as a helicase. Notably, the polarity of the Mre11 exonuclease activity (3'-5') is in opposition to the requirement for forming the 3' overhangs necessary for the initiation of homologous recombination. Homodimers of both Mre11 and Rad50 interact to form a complex referred to hereafter as the MR complex. The MR complex in eukaryotes also includes the Nbs1 (human)/Xrs2 (*Saccharomyces cerevisiae*) protein. Xrs2 binds DNA and Mre11, and aids in the localization of the complex to the DSB as well as stimulation of the Mre11 exonuclease (Symington 2002). Yeast MR complex mutants are sensitive to ionizing radiation and exposure to alkylating agents (Symington 2002), and have a slow-growth phenotype (Shor et al. 2002). Mre11 has been shown to be required for complex formation with Rad50 and Xrs2 (Symington 2002). Deletion of *mre11* or *rad50* can be complemented by overexpression of the ExoI 5'-3' exonuclease in yeast (Lewis et al. 2002). Conversely, deletion of both *mre11* and *exoI* increases the radiation-sensitive phenotype when compared to the yeast *mre11* single mutant (Moreau et al. 2001). The structure and activity of archaeal Rad50 and Mre11 were examined in *Pyrococcus furiosus*, a hyperthermophilic archaeon (Hopfner et al. 2001; Hopfner et al. 2000a). The data confirmed the structural conservation of MR complex homologs through all three domains of life. In thermophilic archaea, the genes encoding Rad50 and Mre11 are also found in an operon with genes coding for a 5'-3'

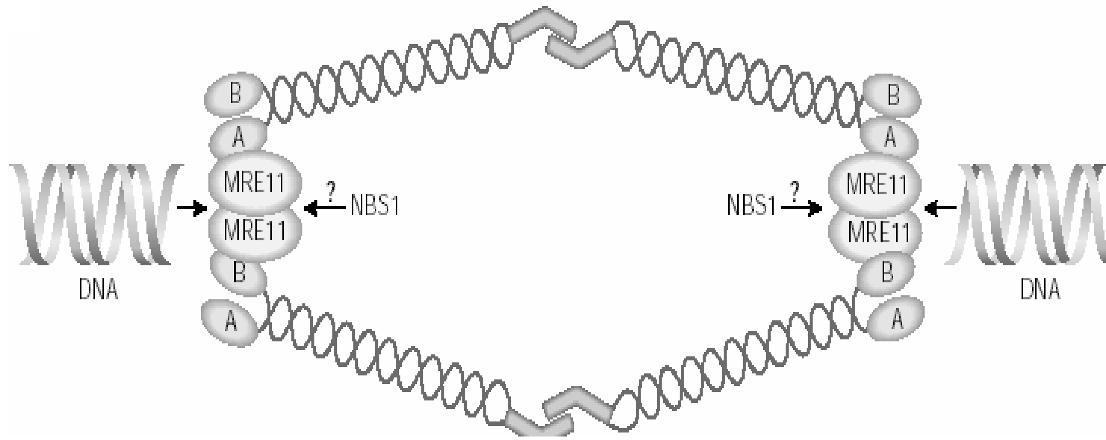


Figure 4-1. Proposed structure of the Mre11/Rad50 complex in Eukarya (human) taken from (van den Bosch et al. 2003). Mre11 is shown in a heterotetramer with Rad50 (Walker A and B motifs of Rad50 shown as 'A' and 'B'). The Nbs1 protein is thought to act in a complex with Mre11 and Rad50 by enhancing Mre11 nuclease activities.

nuclease (*nurA*) (Constantinesco et al. 2002), and helicase (*herA/mla*)(Constantinesco et al. 2004; Manzan et al. 2004). Interestingly, only genes encoding Rad50 and Mre11 are present in the genome of the mesophilic archaeon, *Halobacterium* sp. str. NRC-1 (*Halobacterium)*.

Halobacterium is an extreme halophile growing optimally in 4M NaCl (DasSarma and Fleischmann 1995). Intracellular salt, 4M KCl (Engel and Catchpole 2005), is used by this organism in place of compatible solutes to counter-balance the high external salt concentration. The exceptional ability of *Halobacterium* to survive extremely high level of ionizing radiation has been attributed to adaptations to hypersaline environments characterized by high levels of solar radiation and periodic desiccation (Kottemann et al. 2005; Whitehead et al. 2006). *Halobacterium* has up to 25 copies its genome during log-phase growth and 15 copies during stationary phase (Breuert et al. 2006) potentially providing homologous DNA for recombination-repair pathways. The free-radical scavenging capability of membrane pigments, specifically bacterioruberin, has been shown to provide *Halobacterium* with protection against cellular damages by ionizing radiation (Kottemann et al. 2005; Shahmohammadi et al. 1998). Whole genome transcriptional analysis has suggested that HR is the major pathway for the repair of DSBs in *Halobacterium* (Whitehead et al. 2006) and homologs of eukaryotic HR proteins have been identified in its genome (Ng et al. 2000) (Table 4-1). The *Halobacterium* genome lacks homologs for genes encoding the Ku70 and Ku80 proteins involved in NHEJ pathway for repair of DNA DSBs, thereby reducing the potential pool of alternative repair systems for DSBs.

	ORF Identity in *Halobacterium*	Structural Features	Activity	Role in Homologous Recombination Repair of DNA DSBs
Mre11	VNG0512G	homodimer; complex with Rad50, phosphoesterase domain and two DNA binding domains, Mn cofactor required	DNA binding, DNA duplex unwinding, ssDNA&dsDNA endonuclease, 3'-5' dsDNA exonuclease	DNA DSB recognititon and creation of a 3'-OH overhang, also involved in recombinase loading
Rad50	VNG0514C	homodimer; complex with Mre11, Walker A and Walker B motifs, coiled coil motifs for dimerization separated by a hook motif using Zn+	ATP-dependant DNA binding, ATPase activity	
RadA (also called RadA1)	VNG2473G	homopolymer; Walker A for ATPase and Walker B motifs for ion binding, helix-hairpin-helix motif	Recombinase, homolog of Rad51 (Eukarya) and RecA (Bacteria)	Homologous strand invasion and exchange
RadB (also called RadA2)	VNG1665G	N terminal domain found in RadA & Rad51 missing in RadB	Paralog of RadA lacking in recombinase function *in vitro*	Possible regulatory functions as an ATP-dependant Hjr repressor
Hjm (also called Rad24b)	VNG2368G	DEAD box helicase domain	monomeric ATP-dependant DEAD box helicase	Holliday junction branch migration
Hjr (also called Hjc)	VNG2252G	homodimer	nuclease	Holliday junction resolution
Hef (also called Rad1/XPF/Eif4a)	VNG2356G	homodimer, DEAH box helicase domain at N-terminus in Euryarchaeota (PCNA in Crenarchaeota), XPF endonuclease	Helicase/endonuclease for forked structures	Reverse branch migration for restoration of replication fork
Flap (also called Rad2/XPG/ExoI)	VNG1359G	monomer, helical clamp and PCNA binding region	nuclease	Endonuclease involved in BER long patch repair (DSB formation during post-irradiation repair)

Table 4-1. Structure and function of known archaeal HR repair proteins.

Here *Halobacterium* is used as a model system for archaea and for a genetic approach to investigate the cellular roles of Rad50 and Mre11 in the repair of DNA DSBs and in the radiation resistance of this microorganism. Our phenotypic analysis of *rad50*, *mre11* and *mre11*/*rad50* knockout mutants did not show increase sensitivity to ionizing radiation, UV or the alkylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG). This is in contrast to previous studies with yeast Rad50 mutants that are highly sensitive to irradiation, MNNG and UV, all conditions producing DSBs. We also show a delay in the repair of DSBs with *mre11* and *rad50/mre11* mutants.

<u>*Results*</u>

Targeted Gene Deletion of *mre11* and *rad50*

To determine whether the Rad50 and Mre11 proteins encoded in the genome of *Halobacterium* are involved in the repair of DNA DSBs we carried out targeted gene deletions of the *rad50* and *mre11* genes (single and double deletions) using a modified gene deletion system (see Materials and Methods). For this study, an auxotrophic strain of *Halobacterium* deficient in the biosynthesis of both tryptophan and uracil (Δ*trpA*Δ*ura3*) was constructed for use as a background strain for the targeted gene deletions (Table 4-2). This double auxotroph allowed the use of high-salt drop-out media developed in this study for selection thereby reducing the final pool of colonies obtained to those containing at least one copy of the mutant genome in each cell. The *mre11* and *rad50* genes are located together in an operon on the

Strain	**Genotype**	**References**
Δ*ura3*	Δ*ura3*	{Whitehead, 2006 444 /id}
Δ*trpA*	Δ*trpA* Δ*ura3*	This work
Δ*mre11*	Δ*mre11* Δ*trpA* Δ*ura3+ trpA*	This work
Δ*rad50*	Δ*rad50* Δ*trpA* Δ*ura3+ trpA*	This work
Δ*mre11*Δ*rad50*	Δ*mre11* Δ*rad50* Δ*trpA* Δ*ura3+ trpA*	This work

Table 4-2. *Halobacterium* strains used in this study.

Halobacterium main chromosome with the *mre11* coding region positioned upstream of the *rad50* coding region.

Copies of both wild type and deleted genes were identified by PCR after completing one round of targeted gene deletion for *mre11*, whereas a full deletion for *rad50*, was achieved after the first attempt. Multiple rounds of transformations of clones containing both wild type and Δ*mre11* chromosomes with the Δ*mre11* gene deletion construct finally allowed for complete deletion of *mre11*, found upstream of *rad50* together in an operon. The double mutant (Δ*mre11*/Δ*rad50*) was constructed using the Δ*rad50* mutant strain. The genotypes of the Δ*rad50*, Δ*mre11*, andΔ*mre11*/Δ*rad50* mutant strains were confirmed by Southern hybridization (Figure 4-2) after initial screening by PCR. Reverse transcription of mRNA transcripts followed by PCR amplification of both *mre11* and *rad50* genes (Figure 4-3) provided evidence that deletion of one gene in the *mre11-rad50* operon did not affect transcription in the other.

Growth and Survival of *mre11* and *rad50* Mutants

Phenotypic characterization of the Δ*rad50,* Δ*mre11,* and Δ*mre11*/Δ*rad50* mutant strains revealed no temperature dependant growth effects (data not shown), but did show a slower, but reproducible, growth rate under standard culturing conditions compared to the background strain (Figure 4-4). The 3 mutant strains showed the same level of resistance to the alkylating agent MNNG as the background strain (Figure 4-5A). In contrast, a decrease in survival was observed in both the Δ*mre11*

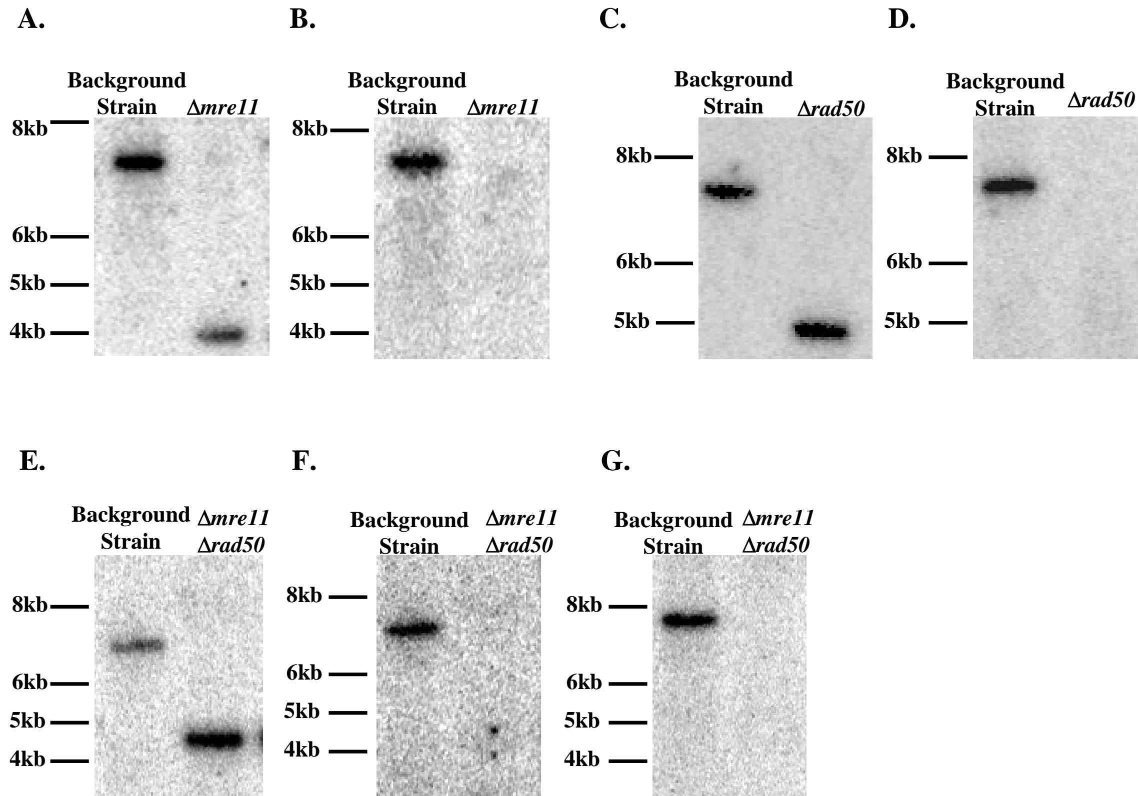


Figure 4-2. Southern hybridizations showing gene deletions for Δ*mre11*, Δ*rad50*, and Δ*mre11*Δ*rad50*. Probes were designed to hybridize to regions 500bp upstream the *mre11* coding region (**A**), 500bp downstream of the *rad50* coding region (**C**) and (**E**), and within the coding regions of *mre11* (**B**) and (**G**) and *rad50* (**D**) and (**F**).

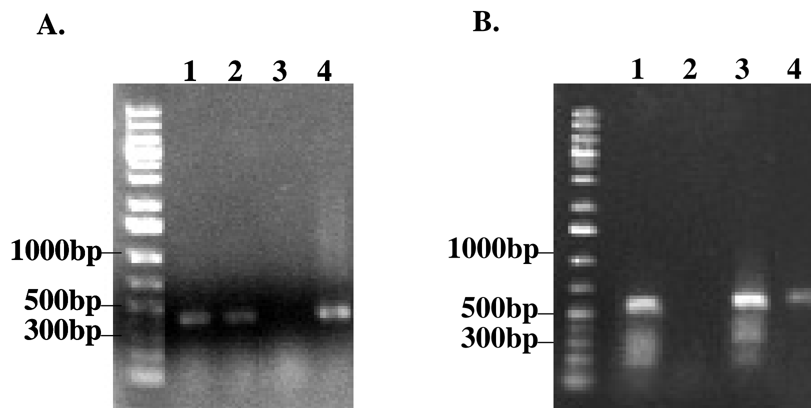


Figure 4-3. Reverse transcription PCR showing production of mRNA transcripts in the background strain (lane 1), Δ*rad50* (lane 2), and Δ*mre11* (lane 3) strains, with a wild-type *Halobacterium* DNA positive control (lane 4). Transcripts for *mre11* were identified in the background strain as well as Δ*rad50*, but lacking in Δ*mre11* as expected (**A**). Transcripts for *rad50* were identified in the background strain as well as Δ*mre11*, but lacking in Δ*rad50* as expected (**B**), showing that deletion of the first gene in the operon (*mre11*) does not result in failure to transcribe the entire operon.

and Δ*mre11*/Δ*rad50* after exposure to 200J/m^2 of UV-C (254nm) radiation which was eliminated at the higher dose of 350J/m^2 when compared to the level of survival of the background strain (Figure 4-5B). Holding the cells in liquid recovery for up to 1 hour did not change the level of survival of the mutants versus background strains, eliminating the possibility of plating as a compounding stress for the mutants strains. Surprisingly, no differential survival in any of the mutants was observed after exposure to 2.5kGy of γ–radiation, even after a second 2.5kGy dose following a 4hr incubation at 42°C with shaking, to allow time for minimal repair of DSBs in the background strain (Figure 4-5C,D). Sensitivity to ionizing radiation is the hallmark of mutations to Mre11 and Rad50 in eukaryotic systems (Symington 2002). For all treatments, the levels of survival of the background strain were comparable to that of the wild-type ((Baliga et al. 2004; Kottemann et al. 2005)).

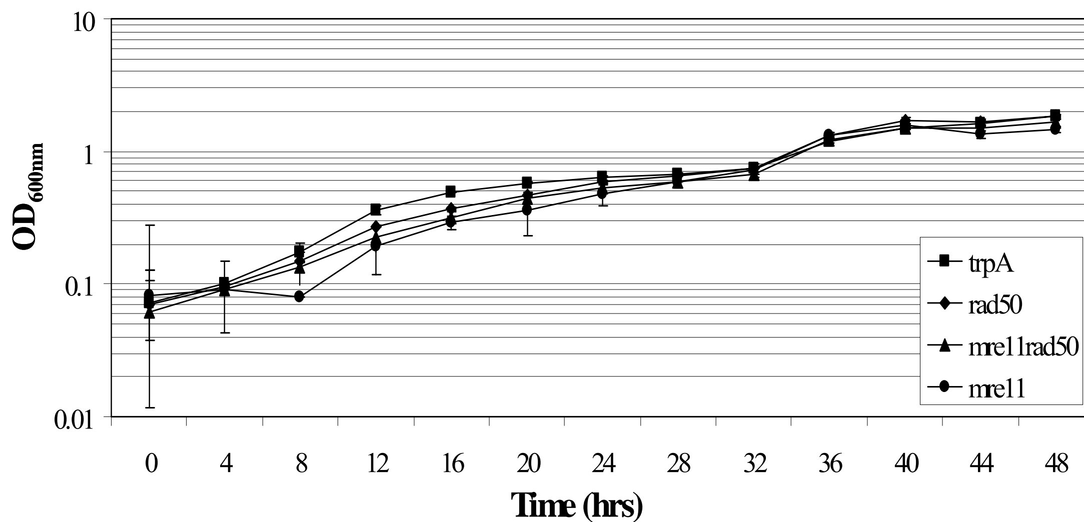
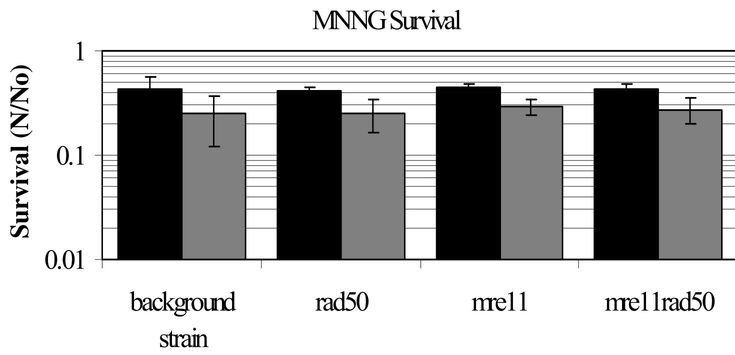
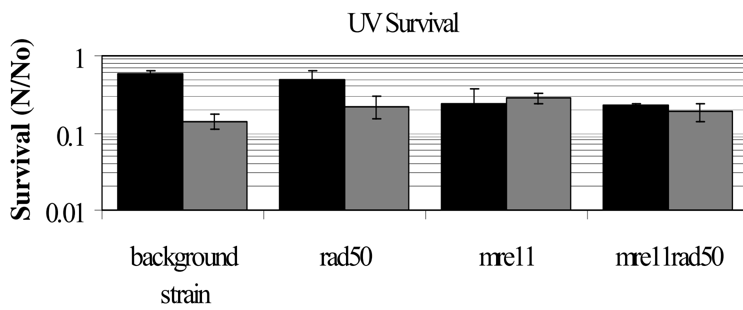


Figure 4-4. Growth curve showing differential growth rate between the background strain (squares), Δ*mre11* (triangles), Δ*rad50* (diamonds), and Δ*mre11*Δ*rad50* (circles) strains. Data shown is the average of at least 2 replicates with standard errors shown.

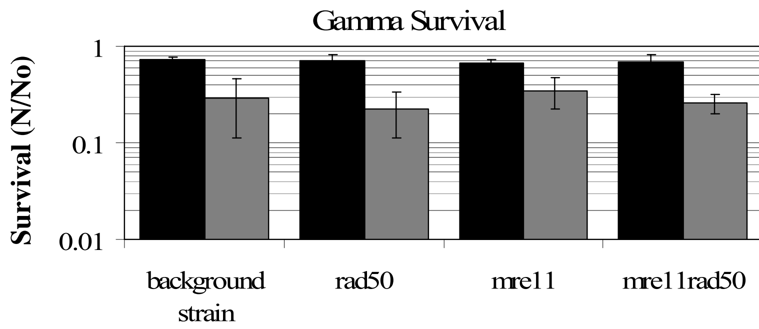
A.



B.



C.



D.

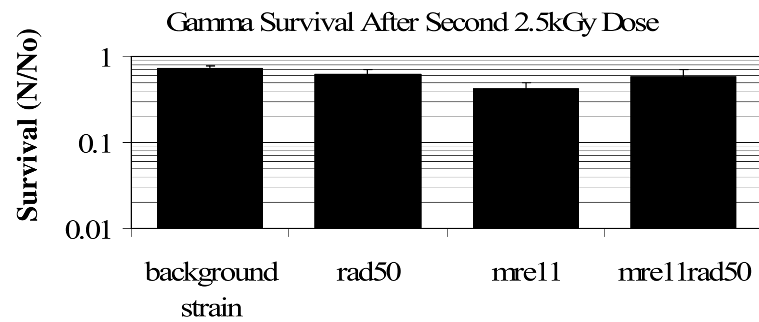


Figure 4-5. Survival of the background strains and the mutants strains Δ*mre11*, Δ*rad50*, and Δ*mre11*Δ*rad50* after exposure to 50ug/mL (black) and 100ug/mL (grey) MNNG (**A**), 200J/m^2 (black) and 350J/m^2 (grey) UV-C radiation with recovery in the dark to prevent photoreactivation repair (**B**), γ-radiation at doses of 2.5 (black), and 5kGy (grey) (**C**), and γ-radiation at a dose of 2.5kGy followed by a 4hr incubation under standard culturing conditions followed by a second dose of 2.5kGy (**D**). Survival was calculated as the average ratio (N/No) of surviving colony forming units from treated (N) compared to untreated (No) cultures. Data shown is the average of at least 3 replicates with standard errors shown. The differences in survival between mutant strains and the background strain were not statistically significant ($P>0.05$) for all conditions with the exception of UV-C irradiation at a dose of 200J/m^2, with Δ*mre11* and Δ*mre11*Δ*rad50* showing significantly decreased survival (P values were 0.0414 and 0.0002, respectively) compared to the background strain. This differential survival was not seen at a dose of 350J/m^2.

DNA Double Strand Break Repair in *mre11* and *rad50* Mutants

The ability to repair DNA DSBs was assayed in each of the mutant strains after exposure to 2.5kGy of γ–radiation, which represents nearly 80% survival in wild-type *Halobacterium* (Kottemann et al. 2005). Samples were taken both prior to irradiation and over a timecourse of recovery under standard culturing conditions. Agarose plugs (0.8%) were made containing $1x10^9$cells/mL and analyzed by pulsed field gel electrophoresis (PFGE). In the background strain and Δ*rad50* mutant, repair of chromosomal fragmentation after exposure to ionizing radiation was initiated within 4hrs and completed within 12hrs (Figure 4-6). In contrast, the recovery process for the Δ*mre11* and Δ*mre11*/Δ*rad50* mutant strains took much longer and those mutants showed no evidence of repair until the 12hr time point. The Δ*mre11* and Δ*mre11*/Δ*rad50* mutant strains did not show increase sensitivity to γ-irradiation at 2.5kGy, implying that the repair of DNA DSBs goes to completion in these strains but at a much reduced rate than that of the Δ*rad50* mutant strain or the background strain. This also suggests that the inhibitory effect on the kinetics of radiation induced-DSBs repair resulted from the absence of Mre11 proteins in the cells.

Homologous Recombination in *mre11* and *rad50* Mutants

To investigate the role of Rad50 and Mre11 in HR, independently of the repair of DNA DSBs and the processing of broken DNA-ends, we developed a plasmid-based recombination assay in *Halobacterium*. A construct was made using a suicide plasmid carrying a wild type copy of the *ura*3 gene, for selection, and 1kbp of non-coding

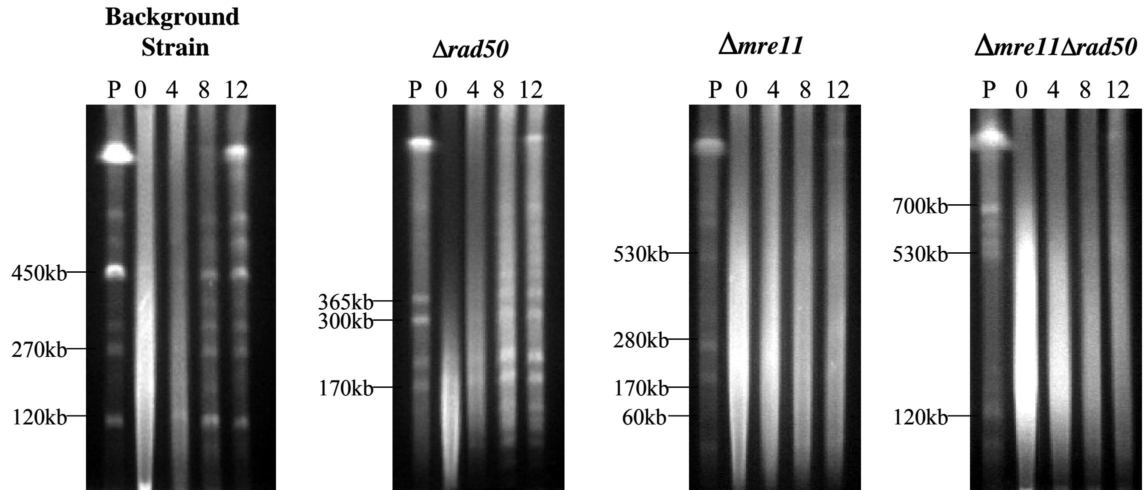


Figure 4-6. PFGE timecourse of recovery after exposure to 2.5kGy of γ-radiation. Samples were taken pre-irradiation (P) as well as immediately following irradiation (0), and every 4hrs during the recovery up to 12hrs (4, 8, 12), embedded in InCert agarose plugs at a final density of $1x10^9$cells/mL. Plugs were digested with *Xba*I prior to gel electrohoresis. Images taken from 1 of 3 independent replicates of this experiment.

region plus 3 nucleotides of coding region for the *mut*S1 gene of *Halobacterium*. Following transformation into Δ*rad50,* Δ*mre11*, and Δ*mre11*/Δ*rad50 Halobacterium* strains along with the background strain*,* recombinants were selected by plating on uracil dropout media.

The results of this assay, shown in Figure 4-7, include intrareplicate variations that decrease the reliability of the data and the ability to interprete small-scale variations in recombination efficiency between strains. This was due to unavoidable variations between replicates inherent in the current standard transformation protocol for *Halobacterium* (Cline et al. 1995) in steps involving the stripping of the S-layer to produce spheroplast cells, the concentration of plasmids and spheroplast cells using polyethelyne glycol, and the resuspension of delicate spheroplast cells after incubation with plasmid DNA. Attempts were made to account for these variations by enumerating total viable cells for each replicate in addition to transformants, and by using 3 pseudoreplicates per transformation (each culture was split into 3 pseudoreplicates) for each of the 3-6 replicates performed for each strain. Negative controls used for each replicate validated the effectiveness of the selection using uracil drop-out medium. This experiment was also repeated in full with 2 additional plasmids bearing alternate target genes (*bop*, *gvpA*) producing the same range of variation, eliminating error based on the target gene. Despite these measures, the standard errors produced did not allow for meaningful inferences from the data. Additional refinements to the transformation protocol reducing variation between replicates will be required before this assay can produce useful data. Nevertheless, the

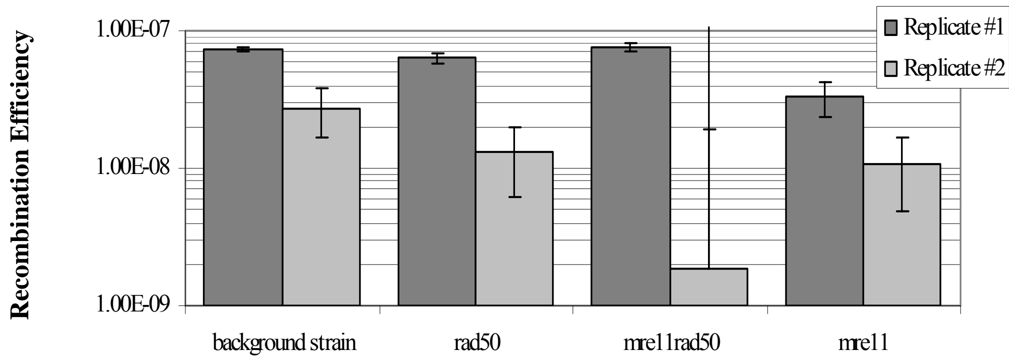
deletion of *rad50* and/or *mre11* did not produce large-scale decreases in the efficiency of homologous recombination.

Discussion

Exposure to reactive oxygen species (ROS), whether by desiccation, exposure to ionizing radiation, or the auto-oxidation of dehydrogenases involved in electron transport as part of aerobic respiration, can result in oxidation of DNA base and sugar moieties and DNA double strand breaks (DSBs) (reviewed in Imlay 2003; Imlay and Linn 1988). DSB production is not limited to the actions of free radical species, however. Base excision repair (BER) of oxidized DNA bases found in clusters (Dianov et al. 2001; Blaisdell and Wallace 2001a) can lead to the formation of cytotoxic DSBs. Homologous chromosomes serve as templates for recombination-repair pathways leading to repair of DSBs, yielding advantages to microorganisms with a multiplicity of genomic material. Radiation-resistant organisms such as *D. radiodurans* have multiple genome copies at all phases of the cell cycle. *Halobacterium salinarium* has been shown to have 25 copies its genome present during log-phase growth and 15 copies during stationary phase (Breuert et al. 2006).

In yeast, a major pathway for the repair of DSBs is homologous recombination (HR), which requires the Mre11/Rad50 (MR) complex (Symington 2002). Mre11 is a 3'-5' dsDNA exonuclease that is regulated through association with Rad50, an ATPase

A.



B.

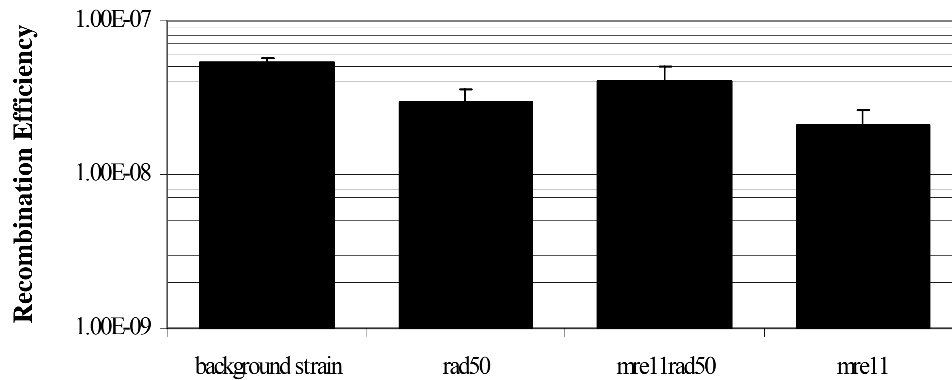


Figure 4-7. Homologous recombination assay data showing transformation efficiency of Δ*mre11*, Δ*rad50*, and Δ*mre11*Δ*rad50* mutant strains of *Halobacterium* compared to the background strain. The recombination efficiency was calculated as the ratio of transformants compared to total viable cells after transformation. Each replicate culture derived from a single colony was divided into 3 pseudoreplicates; each of which was transformed with the *mutS1* pNBK07 plasmid. The intra-replicate variation between pseudoreplicates is shown in **(A)** with standard deviations, and the inter-replicate variation between full replicates is shown in **(B)** with standard errors. Results from two full replicates were used for statistical analysis. The large standard

deviation between pseudoreplicates was derived from variations inherent in the *Halobacterium* transformation protocol, decreasing the reliability of the data despite the reduction in the standard error brought about by increasing the number of replicates.

with DNA binding activity (Assenmacher and Hopfner 2004). The resulting MR complex plays an enzymatic role in DNA-end processing and a structural role in DNA-end joining (Aylon and Kupiec 2004). Orthologs of those proteins, SbcC (Rad50) and SbcD (Mre11) are present in bacterial genomes and have also been implicated in the repair of DNA DSBs (Kowalczykowski et al. 1994; Bentchikou et al., 2007). In the Archaea, homologs of the *rad50* and *mre11* genes have been found in all the genomes sequenced so far, and the corresponding proteins have been biochemically characterized in the hyperthermophile *P. furiosus* (Hopfner et al. 2000a; Hopfner et al. 2001). Here, we investigated the cellular role of Rad50 and Mre11 in a member of the Archaea, the radiation resistant halophile, *Halobacterium*, using a genetic approach. We constructed in-frame deletion mutants of *Halobacterium* for *rad50*, *mre11* and *mre11*/*rad50* and determined the phenotype of the deletion mutants for growth and for survival following DNA damaging treatments.

We found a slight growth defect under standard culturing conditions (42°C with shaking) for Δ*mre11* and Δ*mre11*/Δ*rad50 Halobacterium* mutant strains. This is similar to studies in yeast that show a growth defect for both *rad50* and *mre11* mutants that is stronger in *mre11* strains (Shor et al. 2002). Surprisingly, there was no increased sensitivity to ionizing radiation or MNNG (alkylating agent) for any the *Halobacterium* mutant strains when compared to the background strain. Sensitivity to ionizing radiation is the defining characteristic of MR complex mutants in yeast, along with sensitivity to alkylating agents (reviewed in Symington 2002). Studies

with bacteria also showed increased sensitivity to ionizing radiation for *sbcC* and *sbcD* mutants in *B. subtilis* and in the radiation resistant bacterium *D. radiodurans* (Bentchikou et al. 2007; Mascarenhas et al. 2006).

Using PFGE analysis, we found that *Halobacterium* Δ*mre11* and Δ*mre11*/Δ*rad50* mutant strains displayed extensive delay in the repair of DNA DSBs whereas the Δ*rad50* mutant strain showed similar kinetics of repair compared to the background strain. After 4hrs of recovery after exposure to 2.5kGy of γ-radiation, the background and Δ*rad50* strains began to show some repair of chromosomal fragmentation, whereas strains lacking *mre11* did not begin to display repair of DSBs until the 12hr timepoint. Exposure to a second 2.5kGy dose of γ–radiation 4hrs after an initial dose was expected to result in a significant reduction in survival of the Δ*mre11* and Δ*mre11*/Δ*rad50* strains, which was not observed. This indicates that, despite a reduced rate of homologous recombination repair of DNA DSBs in mutant strains lacking *mre11*, a sufficient reduction in chromosomal fragmentation has occured after 4hrs to allow survival of the strains. This recovery may be the product of multiple genome copies that increase the substrate for recombination repair of DSBs together with an extended recovery period (10 days) to allow for completion of repair in strains lacking *mre11*. Deletion of *D. radiodurans sbcC* and *sbcD* genes also showed a delay in the reconstitution of intact chromosomes as well as delayed growth following γ-irradiation (Bentchikou et al. 2007). The lack of uniformity between *rad50* and *mre11* deletion phenotypes, however, suggests separate functions for Rad50 and Mre11 outside of their activity as a complex, which represents a departure

from both bacterial (SbcC and SbcD) and eukaryotic (Mre11 and Rad50) homologs. In yeast, the MR complex is directly involved in DNA-end processing through the exonuclease activity of Mre11 (Symington 2002). The polarity of Mre11 exonuclease activity, however, is contrary to that required for the formation of 3' overhangs used as substrates for HR, suggesting that another nuclease might be involved in DNA-end processing (Symington 2002). Expression of eukaryotic ExoI, a 5'-3' exonuclease, has been shown to complement both *rad50* and *mre11* deletions in yeast (Lewis et al. 2002).

Conclusion

The absence of a requirement for either Rad50 or Mre11 for DNA repair after exposure to a range of DNA damaging conditions, combined with the lack of dramatic decrease in recombination efficiency in mutant strains lacking *rad50* and/or *mre11*, and the decreased rate of repair of DNA DSBs observed using PFGE analysis leads to either one of two alternative hypotheses: (1) There is no role for the Mre11/Rad50 complex in the homologous recombination pathway in *Halobacterium* but rather an alternative role for the Mre11 nuclease, or (2) There are redundant pathways with reduced efficiency that can operate in the absence of Mre11, specifically. It is not possible based on the data presented here to differentiate between these alternative hypotheses. However, the validity of each hypothesis can be corroborated.

The absence of a distict phenotype based on survival after exposuring to DNA damaging agents and homologous recombination efficiency may indicate a lack of functionality for the Mre11/Rad50 complex in the HR pathway. The presence of some recombination activity based on the recombination assay shown in this study indicates that the HR pathway is functional in the absence of both Mre11 and Rad50. This hypothesis establishes a variance between the archaeal and eukaryotic HR pathway, despite the structural and sequence conservation of Mre11 and Rad50 proteins between the two domains of life. On the other hand, the presence of alternative redundant pathways for a specific type of repair can be substatiated using as an example the RecBDC and RecFOR pathways in *E. coli* for repair of DNA DSBs. In addition, the *Halobacterium* genome encodes homologs of both bacterial (UvrA, UvrB, UvrC, UvrD) and eukaryotic (XPF, XPG, XPD) nucleotide excision repair proteins thereby providing alternative sources of repair proteins for a given pathway. In the case of HR repair of DSBs, any such alternative repair pathway would operate at a reduce rate based on evidence gathered using PFGE analysis and γ-irradiation survival data. Additional analyses will be required to test both hypotheses.

Methods and Materials

Cultures and Growth Conditions

Halobacterium sp. strain NRC-1 cultures were grown in standard GN101 medium [250g/L NaCl, 20g/L MgSO4-7H20, 2g/L KCl, 3g/L Na citrate, 10g/L Oxoid peptone

(pH 7.2) with the addition of 1mL/L Trace Elements Solution (31.5mg/L $FeSO_4.7H_2O$, 4.4mg/L $ZnSO_4.7H_2O$, 3.3mg/L $MnSO_4.H_2O$, 0.1mg/L $CuSO_4.5H_2O$)] (Hackett and DasSarma 1989) at 42°C with shaking at 220 rpm (Gyromax 737, Amerex Instruments; Lafyatte, CA). Tryptophan and uracil drop-out media were made using yeast synthetic drop out media supplements (without tryptophan and without uracil) (1.92g/L) from Sigma (St. Louis, MO) and yeast nitrogen base without amino acids (10g/L) (Sigma; St. Louis, MO) suspended in a basal salt solution [(250g/L NaCl, 20g/L MgSO4-7H20, 2g/L KCl, 3g/L Na citrate; pH 7) with the addition of 1mL/L Trace Elements Solution (31.5mg/L $FeSO_4.7H_2O$, 4.4mg/L $ZnSO_4.7H_2O$, 3.3mg/L $MnSO_4.H_2O$, 0.1mg/L $CuSO_4.5H_2O$)] and 20g/L agar. When specified, uracil was added to a final concentration of 50ug/mL, tryptophan to a final concentration of 50ug/mL and 5- fluoroorotic acid (5-FOA) to a final concentration 0.3mg/ml.

Targeted Gene Deletion

Targeted gene deletions were constructed using the protocol by Peck *et al.* (Peck et al. 2000) using plasmid pNBK07 and the following changes: The gene knockout construct for each gene was composed of 500bp upstream and downstream of the target gene flanking the *trpA* gene for tryptophan biosynthesis under the control of the constitutively expressed ferrodoxin promoter, as described in (Allers et al. 2004). A background strain auxotrophic for both tryptophan and uracil biosynthesis (Δ*trpA*Δ*ura3*) was used in place of the Δ*ura3* strain to allow for the use of uracil dropout medium instead of mevinolin as the selective agent for selection for uracil

prototrophy following transformation with plasmid pNBK07 bearing knockout gene constructs and the *ura3* marker for uracil biosynthesis. Tryptophan dropout medium supplemented with 5-FOA (Sigma; St. Louis, MO) was subsequently used to select intramolecular recombinants that had lost the plasmid and acquired tryptophan prototrophy. Deletions were confirmed by Southern hybridization using probes both within the deleted region and up- or down-stream of the deleted region. In addition, production of a transcript for the *mre11* and *rad50* genes was analyzed to confirm that disruption in one gene in the operon did not affect transcription of the second gene. Total RNA was extracted using the Stratagene Absolute RNA kit (La Jolla, CA), followed by treatment with RQ1 RNase-free DNase (Promega; Madison, WI), and production of cDNA using the TaqMan Reverse Transcription Kit (Applied Biosystems; Foster City, CA). FastTaq PCR reagents (Roche Applied Sciences; Indianapolis, IN) were used to eliminate multiple PCR products due to the high-GC content of the *Halobacterium* genome. PCR products were analyzed by agarose gel electrophoresis.

UV-C and γ-Irradiation Survival

UV-C (254nm) and γ-irradiation survival assays were conducted and quantified as described by Baliga *et al.* (Baliga et al. 2004) and Kottemann *et al.* (Kottemann et al. 2005), respectively. Recovery after UV-C irradiation was conducted in the dark to limit photo-reactivation repair. Survival was measured by the ratio of the total number of viable cells after irradiation (N) divided by the total number of viable cells in the control sample (No).

MNNG Survival

Cultures were grown to log phase (OD_{600nm} = 0.8) in standard GN101 medium at 42°C with shaking prior to being divided into 5 aliquots and diluted to OD_{600nm} = 0.2 in GN101 medium with addition of 50ug/mL and 100ug/mL *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine (MNNG) (2x50ug/mL, 2x100ug/mL, 1xGN101 without MNNG) to a final volume of 5mL. OD values were recorded after approximately 24hrs of incubation at 42°C, averaged, and compared to values obtained for cultures growing in the standard GN101 medium. At least 3 independent experiments were carried out for each strain.

Growth Curves and Temperature-Dependant Growth Assays

Cultures of each deletion mutant strain and the Δ*trpA*Δ*ura3* background strain were grown to log phase (OD_{600nm} = 0.6) together with the background strain, diluted back to OD_{600nm} = 0.05 in GN101 supplemented with tryptophan and uracil, and incubated at 42°C with shaking. OD_{600nm} readings were taken at intervals and recorded. At least 3 independent experiments were carried out for each strain.

Recombination Assay

5mL cultures of each strain (background strain, Δ*rad50*, Δ*mre11*, Δ*mre11*Δ*rad50*) were grown in GN101 supplemented with uracil (50ug/mL) and tryptophan (50ug/mL) to stationary phase (OD_{600nm} = 1.0-1.5) (Cline et al. 1995). 150ng of

plasmid DNA was used in each transformation. The pNBK07 plasmid bearing 500bp upstream and downstream of the *mutS1* coding region was used allowing for homologous recombination at the *mutS1* gene. The *ura3* gene on the plasmid was used as a selective marker for uracil biosynthesis in the formerly Δ*ura3* recombinants as pNBK07 lacks a halophilic origin of replication allowing for replication inside *Halobacterium* cells without integration into the chromosome. Cultures were plated after 80-90% recovery of the rod structure from the spheroplast form. Cells were plated onto GN101 solid medium supplemented with uracil (50ug/mL) and tryptophan (50ug/mL) to determine the total viable cell count in addition to plating on uracil dropout medium to determine the number of recombinant colony forming units. The ratio of recombinants to total viable cells was determined and compared for the background strain and each mutant strain (Δ*rad50*, Δ*mre11*, Δ*mre11*Δ*rad50*).

γ-Irradiation Time Course of Recovery PFGE Analysis

Cultures of *Halobacterium* strains (background strain, Δ*rad50*, Δ*mre11*, Δ*mre11*Δ*rad50*) in GN101 with uracil + tryptophan were grown to OD_{600nm} = 0.6 (log phase, $2x10^8$cells/mL), concentrated by centrifugation and irradiated using a ^{60}Co γ source (University of Maryland College Park γ Test Facility) to a final dose of 2.5kGy (dose rate = 3-13kGy/hr). Cultures were brought back to full volume in GN101 with uracil + tryptophan and incubated at 42°C with shaking for 12 hours. Samples were taken at each of the following timepoint: pre-irradiation, 0hr, 2hr, 4hr, 8hr, 12hr), cells were pelleted by centrifugation at 8000 x g for 5min and resuspended

in room temperature BSS (250 g/L NaCl, 20 g/L $MgSO_4$, 2 g/L KCl, 3 g/L sodium citrate) prior to being embedded into InCert agarose plugs (0.8% final concentration prepared in 3:1 BSS:dIH_2O; BioRad; Hercules, CA) at a final cell concentration of $1x10^9$ cells/mL. Plugs were lysed in proteinase K solution (0.25M EDTA (pH8), 1% N-lauryl sarkosine, and 0.5mg/mL proteinase K at 54°C for 1-2 days. Plug washes consisted of 2x1hr in 20mL 1x TE buffer at room temperature, 2x1hr in 20mL 0.5x TE buffer at room temperature, 4x24hr in 0.5x TE buffer at 4°C. Plugs were stored in 5mL 0.5x TE buffer at 4°C after wash steps. *Halobacterium* genomic DNA plugs were analyzed using a CHEF DR-III electrophoresis system (BioRad; Hercules, CA) using 1% PFGE certified agarose (BioRad; Hercules, CA) gels, 0.25x TBE in both the running and gel buffers. Run conditions were 6V/cm, 10-60s switching times, 120° included angle, for 24hrs at 14°C.

Chapter 5: Conclusions

Conclusions and Perspectives

The effects of ionizing radiation on the extremely halophilic Archaeon *Halobacterium sp. str.* NRC-1 can be divided into three main categories: protection from oxidative damages, response to ionizing radiation, and repair of DNA double strand breaks (DSBs).

Intracellular salts used to maintain osmotic balance in the hypersaline conditions *Halobacterium* cells require were shown in this study to provide protection from oxidative damages through the scavenging of hydroxyl radicals produced from the radiolysis of water by γ-radiation. This represents the first *in vivo* validation of the hypothesis that the presence of chloride compounds can reduce DNA damage from reactive oxygen species. This study also showed that bromide offers even greater protection than chloride to both DNA and proteins after exposure to ionizing radiation. These results highlight both the importance of the intracellular environment in determining radiation resistance and the multiplicity of pathways resulting in radiation resistance that can be utilized by various microbes as a consequence of adaptations to common environmental stresses such as desiccation.

The global stress response to γ-radiation was measured using both full genome microarray analysis of the transcriptional response and iTRAQ analysis of the

proteomic translational response. A global down-regulation of metabolism and stalling of the cell cycle accompanied by an increase in protein turn-over was proposed to allow for repair of DNA fragmentation via the homologous recombination pathway. A 3-fold increase in mRNA abundance was observed for *radA* 30min after gamma irradiation, correlating with an increase in RadA protein abundance. By using both genomic and proteomic analyses, we were able to identify key genes involved in the repair of oxidative damage in *Halobacterium* for further analysis, as well as establish putative identifications of ORFs showing differential expression. Differential regulation of *tfbB, tfbF* and *tfbG* encoding transcription regulatory elements in response to several stressors, as has been observed under other stress conditions (Baliga et al. 2004; Kaur et al. 2006), drives the hypothesis that these three TFBs coordinate various aspects of a cellular stress response (Facciotti et al. 2007). This study adds to the growing library of genomic and proteomic data for *Halobacterium* sp. str. NRC-1 under a variety of environmental stresses generating an excellent model system for studying general stress response in the Archaea.

The role of the Mre11-Rad50 complex was examined in an Archaeal model system to determine if these proteins performed the same role in homologous recombination repair of DSBs as their Eukaryotic homologs. In Eukaryotes, the Mre11 complex functions in DSB recognition and resection of the strand ends, as well as having a role in the creation of a 3'-OH overhang and recruitment of recombinase and other factors required for homologous strand invasion. Deletion of *mre11* in *Halobacterium* was

found to reduce the rate of DSB repair, but not the overall survival of the cells. Two alternative hypotheses can be drawn from the data: (1) the Mre11/Rad50 is not an integral component of the homologous recombination pathway in *Halobacterium* with Mre11 having a yet-to-be-determined role affecting recombination efficiency and growth rate, or (2) there is a redundant pathway for repair of DNA DSBs in *Halobacterium* with reduced processivity.

Taken together, the data presented here provide a halophilic model for radiation resistance that shares some common elements with other radiation resistant organisms such as *D. radiodurans* while presenting alternative mechanisms specific for extreme halophiles. *Halobacterium* also provides a platform for studying the *in vivo* function of DNA repair proteins using genetic techniques not practically applicable in other archaea. The data derived from these studies can be extended to related systems in the Eukarya for which key proteins have yet to be identified.

Future Work

Ionizing radiation results in similar oxidative damages to cellular macromolecules including DNA and proteins as desiccation. Desiccation is an environmental stress common to the hypersaline environments inhabited by *Halobacterium* sp. str. NRC-1. Therefore, comparing the formation and repair of oxidative lesions in DNA bases and proteins as well as DNA double strand breaks (DSBs) by desiccation to damages induced by ionizing radiation is a logical extension of the work presented here. Examination of oxidative damages to other cellular macromolecules such as lipids

would further clarify the source of the cytotoxic effect of ionizing radiation. Cross-analysis of the global stress response to desiccation to the response to ionizing radiation presented here would add to understanding the relationship between adaptations to desiccation and radiation resistance. *In vivo* examinations of other proteins in the homologous recombination repair pathway, including the RadA recombinase and archaeal-specific Hjr and Hjm proteins, should also be done to further understand the HR repair pathway in the Archaea as well as provide a model for understanding the pathway in more complex eukaryotic systems. Finally, the role of the intracellular milieu in protecting cellular components from stress damages should be examined, particularly in the case of organisms such as *Halobacterium* that alter their internal chemical composition to mirror their external environment.

Appendices

Appendix 1. Chapter 3 Supplementary Information

Multiple mechanisms are triggered to minimize and repair γ radiation damage in Halobacterium NRC-1

Below we discuss in more detail the specific aspects of the γ radiation response involved in repair or avoidance of damage including (A) restoration of genome integrity, (B) modulation of dehydrogenases, redoxins and cytochromes to minimize ROS reactions, (C) inhibition of cell division, and (D) coordination of a response regulatory circuit.

A. DNA Repair: Glycosylase Activity and Homologous Recombination

Oxidative damage to nucleotides (Slupphaug et al. 2003) can result in deleterious mutations during subsequent DNA replication. Damaged nucleotides are recognized and removed by glycosylases as part of the base excision repair (BER) pathway. Transcriptional upregulation of the DNA glycosylase Ogg was observed during the *Halobacterium* NRC-1 response to γ radiation as well as an increase in Gap expression. While Ogg is an A/G specific DNA glycosylases function in BER based on primary sequence matches (COG0122; PF00730), Gap, on the other hand, is believed to participate in many cellular processes and has been shown to have uracil

DNA glycosylase activity in eukaryotes (Meyer-Siegler et al. 1991; Wang et al. 1999) and may have a similar multifunctional role in haloarchaea.

Archaeal proteins of the Homologous Recombination repair (HR) pathway are structurally and functionally similar those eukaryotes (Allers and Ngo 2003). The steps in HR repair include detection of the strand break and end processing, strand invasion and formation of heteroduplex DNA, branch migration and resolution of the Holliday junction. Similar to previous observations in bacteria (*recBCD)* (Liu et al. 2003) and eukaryotes (*mre11*/*rad50*) (Gasch et al. 2001), the two putative subunits of the complex for DSB detection and processing in *Halobacterium* NRC-1, VNG0512G (*mre11*) and VNG0514C (*rad50*), were not differentially regulated after γ irradiation. Of the two RecA/Rad51 homologs in archaea, RadA (also called RadA1) and RadB (also called RadA2), only RadA can catalyze strand exchange (Komori et al. 2000a). In *Halobacterium* NRC-1 only RadA1 mRNA and protein levels increased during early γ response which parallels similar DNA damage-responsive regulation of this gene in other organisms (Liu et al. 2003). Likewise, of the two putative branched structure-specific endonucleases (*hjr*, Holliday junction resolvase and *hef*, nuclease/helicase) only *hjr* was up regulated during the γ radiation response.

B. Mechanisms to Minimize Oxidative Damage

Electron transport systems are especially sensitive to increased ROS production (Imlay 2003). Downregulation of 8 dehydrogenases (e.g. *adh2*, *adh3*, *sdh* and *mdh*),

as was observed during halobacterial γ response and may reflect the depletion of intracellular reducing equivalents during severe oxidative stress (Golden and Ramdath 1987) and an attempt to minimize ROS production by further oxidation reactions (Imlay 2003). Increased protein abundances for superoxide dismutase Sod2 might serve to scavenge the free radicals.

C. Coordinated Expression between Cell Division and DNA Replication and Repair Genes

Communication between DNA replication, repair and cell cycle progression is imperative to maintain genomic stability (Sancar et al. 2004). Minichromosome maintenance proteins (Mcm) play essential roles in replication, and in humans, Mcm proteins appear to be recruited to HR sites by hRad51/52 interactions to initiate replication for repair (Shukla et al. 2005). The functional similarity between archaeal Mcm/RadA1 and human Mcm/Rad51 (McGeoch et al. 2005; Seitz et al. 2001), and the upregulation of both components in *Halobacterium NRC-1* after γ irradiation are suggestive of a similar repair relationship in archaea (McGeoch et al. 2005). Furthermore, an inverse relationship was observed between mRNA changes of Mcm and Cdc48c, a CdcH ortholog putatively involved in cell division (Figure 3-3, bottom panel). This relationship is also observed under several other stress conditions (Kaur et al. 2006; Baliga et al. 2004). This observation implies a pause in the cell division cycle as has been observed in other organisms (Sancar et al. 2004; Rieger and Chu 2004; Bridges 1995) and is believed to ensure completion of DNA repair prior to cell division.

D. The γ Response Regulatory Network

Transcriptional regulation in haloarchaea is an amalgam of eukaryotic basal machinery and bacterial transcription regulators (Geiduschek and Ouhammouch 2005). The archaeal preinitiation complex consists of a homolog to eukaryotic RNA polymerase II enzyme (RNAP), TATA-binding proteins (TBPs) and Transcription Factor IIB homologs (TFBs) (Geiduschek and Ouhammouch 2005). Transcription is further modulated by regulatory proteins orthologous to bacterial regulators (Sivaraman et al. 2005) of which there are approximately 130. During the γ response at least 9 regulators were upregulated along with one TBP (*tbpE*) and two TFBs (*tfbB* and *tfbF*). Downregulation of 5 regulators was observed, two which were of unknown function and have been newly annotated (Supplemental Table 3 in (Whitehead et al. 2006)). Besides the 5 regulators, one TFB, *tfbG*, was also repressed during early gamma response.

We have used a protein-DNA interaction map for Halobacterium (Facciotti et al. 2007) to create a rudimentary regulatory network for the γ response to better understand the interaction between TFBs and protein regulators. Binding sites for TfbB, TfbF, TfbD or TfbG were identified upstream of 45% of the 216 γ responsive genes (Supplemental Table 5 in (Whitehead et al. 2006)). The transcription factors bind promoters of over half of the regulators and protein kinases that were also differentially regulated during the γ response, several of which are also differentially

regulated in response other stressor (Baliga et al. 2004; Kaur et al. 2006), indicating a central role in regulating stress responses.

Appendix 2. Chapter 4 Supplementary Information

Attempted Mutations

Targeted gene deletions were attempted for each gene in the hypothetical HR repair pathway in *Halobacterium* outlined in Table 4-1 using the modified gene deletion system described in the Methods and Materials section. *Halobacterium* cells contain multiple copies of the genome in each cell (Breuert et al. 2006). Combined with a deficiency of selective markers available for Archaeal systems, targeted gene deletions are more complex than in Bacterial model organisms. Single gene deletions of *radA*, *radB*, *hjr*, *hjm*, *hef* and *flap* were abandoned after attempts at full gene deletions resulted in strains containing a mix of wild-type and mutant chromosomes as revealed by PCR and/or Southern hybridization assays.

Overexpression of Flap was then attempted based on evidence from yeast showing deletion of the *flap* homolog (FEN-1) results in a fairly γ-resistant strain whereas overexpression results in increased DSB formation after exposure to ionizing radiation (reviewed in Liu et al. 2004). Analysis of this mutant was abandoned after failures to produce a phenotype using MNNG, UV-C radiation, and gamma radiation. Dominant negative overexpression of RadA was attempted in lieu of *radA* deletion after repeated failures to produce the deletion mutant. A conserved lysine in the Walker A motif of *radA* was replaced with an alanine, glycine, or leucine residue and inserted into the pNBPA overexpression plasmid (gift of Dr Nitin Baliga) containing

a halophilic origin of replication. Each of the 3 variants of the dominant negative (*radA*negK256A, *radA*negK256G, *radA*negK256L) mutation along with a plasmid containing a wild-type sequence of *radA* to be used as control during phenotypic analyses were transformed into wild type *Halobacterium* cells using the *hmgA* gene for mevinolin resistance as a selective marker for plasmid uptake. None of the dominant negative strains (*radA*negK256A, *radA*negK256G, *radA*negK256L) showed differential survival after exposure to either 50μM or 100μM of MNNG compared to the background strain. *radA*negK256G and *radA*negK256L both showed decreased survival after exposure to UV-C radiation (200J/m^2, dark recovery) which was unexpected as UV-C radiation produces pyrimidine dimers which are not repaired using a recombinase such as RadA. The survival of *radA*negK256A after exposure to 2.5kGy of gamma radiation was slightly increased compared to the wild-type strain, although no significant differences ($P>0.05$) in survival were observed after exposure to 5kGy of γ-radiation. Analysis of these mutants was discontinued in favor of the Δ*mre11*, Δ*rad50*, and Δ*mre11*Δ*rad50* mutants.

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