CELLULAR RESPONSE OF THE HYPERTHERMOPHILIC ARCHAEON *SULFOLOBUS SOLFATARICUS* TO RADIATION DAMAGE.

By

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CELLULAR RESPONSE OF THE HYPERTHERMOPHILIC ARCHAEON

SULFOLOBUS SOLFATARICUS TO RADIATION DAMAGE.

Abstract

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DNA repair pathways in archaea such as *Sulfolobus* species have long been a source of interest due to natural environments in which they are exposed to high temperatures, acidic pH, and solar radiation. Despite harsh conditions that likely lead to constant DNA damage, these organisms have similar mutation rates to mesophilic species such as *E. coli* and thus are hypothesized to have efficient methods of repairing DNA damage. Here, the response of *Sulfolobus solfataricus* to ultraviolet (UV) and ionizing radiation is investigated. The recovery of *S. solfataricus* to both forms of radiation was found to be strain specific. Investigation of genes involved in the *S. solfataricus* homologous recombination (HR) pathway revealed upregulation of *radA* and *rad54* transcripts following UV and ionizing radiation. Further examination of the *rad54* gene locus elucidated strain specific differences in this gene, and western hybridization analysis only detected a protein product in the strain containing the undisrupted gene. This is the first report of strain specific differences in the *S. solfataricus* response to damage, the first detailed report of increased transcript abundance of genes involved in HR following UV radiation, and the first characterization of SsoRad54 *in vivo*. Our findings about the *S. solfataricus* damage response are consistent with the precedent of this organism as a model archaeon for studying complex eukaryotic processes such as DNA repair.

To gain a better understanding of double-strand break repair mechanisms in archaea, the *S. solfataricus* genome was searched for evidence of an end-joining pathway. An uncharacterized open reading frame was identified as a putative *S. solfataricus* Ku gene. Transcription of this gene was found to be upregulated in response to UV and ionizing radiation, indicating potential involvement in DNA repair. Finally, *S. solfataricus* whole cell extracts were assessed for end-joining activity and found to consistently ligate linear DNA substrates. Together, these results implicate a previously uncharacterized end-joining pathway in *S. solfataricus*.

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To Jacob and Ransom Laughery. May what I have learned help me to be not only a better scientist, but also a better wife and mother.

CHAPTER ONE
INTRODUCTION

1.1 Archaea are unique model organisms.

1.1.1 Members of the domain Archaea *are distinct from bacteria and eukaryotes.*

Archaea are a group of single celled organisms that are distinct from both eukaryotes and eubacteria (Figure 1.1). Due to phenotypic similarities, they were grouped with bacteria until advances in molecular biology, such analysis of 16S and 18S ribosomal RNA, led to the discovery that they are no more similar to bacteria than they are to eukaryotes on the molecular level[1]. Finally, in 1990, archaea were separated into their own domain of life[2]. At this time the domain *Archaea* was further divided into two phyla: *Euryarchaeota*, a diverse group including halophiles, methanogens, and several thermophilic species of archaea, and *Crenarchaeota*, a phylum containing many thermophilic species but also a growing set of mesophiles, most of which have been identified in marine environments[2,3]. Since then, two other phyla, *Korarchaeota* and *Nanoarchaeota,* have been added to the growing domain[4-6]. The former phylum was identified from ribosomal RNA sequence analysis of samples taken from a hot spring in Yellowstone National Park, however the organisms themselves have not been isolated[4,5]. A single nanoarchaeal species has been recently identified and lives in a symbiotic or parasitic relationship with a thermophilic crenarchaeal species; future investigations will likely shed light on the characteristics of this new branch and the diversity that they bring to the archaeal domain[6,7].

Archaea have been a source of interest, especially from an evolutionary perspective, because they have both bacterial and eukaryotic-like features (Figure 1.2). It is true that they have similar characteristics to the other domains of life, but considering archaea to be a mere mixture of bacterial and eukaryotic features overlooks unique characteristics found in this branch and none other. Specifically, many archaeal species are notorious for living in extreme habitats such as high salt concentration, high methane

concentration, pH extremes (both acidic and basic), and temperature extremes (both high and low) that few or no other organisms have been found to inhabit (Figure 1.3)[8]. The ability to withstand extreme conditions has made archaea useful in a wide variety of applications including bioremediation, industrial food and paper preparation, clothes detergent, and scientific research[8,9].

Despite these unique features, it is understandable why archaea and bacteria were phylogenetically grouped together for many years. Archaea, like bacteria, are prokaryotes, lacking a true nucleus and membrane-bound organelles. Like bacteria, they are also strictly unicellular, maintain a circular genome, and arrange genes in operons. Traditionally, archaea have also been considered to have similar metabolic, energy conversion, and biosynthetic pathways to bacteria[8,10]. More recent studies challenge this, however, as bioinformatic analyses reveal that archaea have completely different metabolic network design from bacteria; from a molecular perspective, they also often utilize very different enzymes to catalyze metabolic reactions[11,12].

Archaea also bear undisputable similarities to eukaryotes. Archaeal and eukaryotic core enzymes resemble each other in many information processing pathways including replication, transcription, translation, recombination, and some methods of DNA repair; in addition to enzyme similarities, many mechanistic details of these pathways are similar as well[8,13,14]. Similarities also exist in methods of chromosomal packaging. Primitive histone proteins have been found in euryarchaea, and transcriptional regulation of chromatin via acetylation/decetylation of lysine residues of chromatin binding proteins has been documented in crenarchaea (reviewed in[15]). These similarities allow archaea to serve as simple model systems of eukaryotic processes.

1.1.2 The precedent for utilizing archaea as model organisms

The use of archaea as model systems for complex eukaryotic processes has been well established. In addition to similarities between archaeal and eukaryotic systems, many archaeal enzymes

are easier to purify and manipulate due to robustness and stability at room temperatures. Archaeal crystal structures and sequence analyses have given numerous insights into eukaryotic homologues, especially in the field of DNA repair. Elucidation of the *Pyrococcus furiosus* Mre11/Rad50 crystal structure gave structural information and implicated mechanistic roles of the complex in ATP dependent DNA processing and structural linkage of sister chromatids during double-strand break repair[16]. The crystallization of full length *P. furiosus* RadA, the central strand exchange protein for homologous recombination (HR), with and without a fragment of human BRCA2 gave insights into the BRC2-dependent depolymerization of Rad51 complexes following DSB induction, as well as structural information about Rad51 polymerization and interactions with other HR accessory factors[17]. Finally, comparison of eukaryotic and archaeal sequences has led to scientific advances, such as the identification of Spo11 as the initiator of double-strand breaks in meiotic recombination[18]. These are just a few examples of how archaea have been used to gain insight into eukaryotic enzyme structures and mechanisms.

1.2 *Sulfolobus solfataricus*, a thermoacidophilic archaeon, is an ideal model archaeon.

1.2.1 Sulfolobus solfataricus *is well suited for use in a laboratory setting.*

One archaeal species that has served as an excellent model organism is *Sulfolobus solfataricus*, a crenarchaeal thermoacidophile that can be cultured in a laboratory setting at 80°C and pH 3[19]. It is an obligate aerobe and can be grown both in liquid culture or on solid media to allow isolation of single colonies[19]. In addition to the ease of culturing this organism, its complete genome (approximately 3 Mbp) has been sequenced and its proteome has been analyzed[20-22]. A variety of genetic techniques that are unavailable in many other archaea can be performed in this organism. Successful transformation methods have been developed which utilize selectable markers, and natural plasmids and viruses have been adapted to create *S. solfataricus-E. coli* shuttle vectors and *S. solfataricus* expression vectors with inducible promoters (reviewed in [23]). Other available tools include the development of reporter gene constructs and

the ability to perform genetic disruptions[24,25]. *S. solfataricus* is one of the most well studied crenarchaea and its ideal use as a model organism is due in part to the amount of information available about it that is lacking in similar species.

1.2.2 Sulfolobus *is a model archaeal genus with many eukaryotic-like features.*

Sulfolobus has a standing precedent as a model archaeal genus. Diverse studies in *Sulfolobus* ranging from carbon metabolism to translation initiation have benefited our understanding of all archaea[26,27]. Moreover, many foundational studies have led to the realization of striking parallels between *Sulfolobales* and eukaryotes. Early studies of *Sulfolobus* transcription machinery highlighted similarities between archaeal and eukaryotic RNA polymerases (RNAPs)[28]. Archaeal RNAPs are similar to their eukaryotic homologues in complexity: archaeal RNAPs have as many as thirteen subunits, four of which are homologous to the core eukaryotic and eubacterial subunits (although the archaeal proteins are more similar to the former than the latter), and five others that are homologues of eukaryotic subunits but apparently missing in eubacteria[28].

Additionally, the genus *Sulfolobus* has the best characterized archaeal cell cycle[29]. Proteins involved in the replication of the *Sulfolobus* genome appear to be homologous but simplified versions of those found in eukaryotes[30]. This has led to insights into replication mechanisms in eukaryotes that have been otherwise hard to study due to the difficulty of working with their enzymes. One example is the structural resolution of the *S. solfataricus* heterodimeric core primase which is responsible for synthesizing RNA primers for DNA replication[31]. This structure provided useful information about primase mutant phenotypes in eukaryotes because it revealed the nature of the heterodimer interface. Archaeal replication also proceeds from multiple origins along its circular genome similar to eukaryotic replication along linear chromosomes, a characteristic that was initially thought to distinguish prokaryotes from eukaryotes[29,32,33]. Furthermore, the rate of replication in *S. solfataricus* is approximately 10 fold lower than the rate of bacterial

replication and more similar to the rate of eukaryotic replication[32]. Cell sorting has been a useful means of investigating the *Sulfolobus* cell cycle because these organisms do not typically form aggregates, and has revealed that this genus is characterized by a short G1 phase, an S phase lasting about one third of the cell cycle, and a relatively long G2 phase enduring roughly 60-65% of the cell cycle[29,34]. Studies of the lengthy G2 phase indicate that replicated chromosomes remain attached to each other for an extended period of time[34]. This is analogous to the attachment of eukaryotic sister chromatics following DNA replication and contrasts to immediate separation of bacterial chromosomes following DNA replication.

Sulfolobus also has unique similarities to eukaryotes in the field of HR. *S. solfataricus* contains the only known prokaryotic homologue of eukaryotic Rad54, an important accessory factor in this pathway [35]. Further study of ssoRad54 will allow comparisons between archaeal and eukaryotic HR to be made that cannot be achieved through other archaea simply because the homologues have not been identified.

1.3 DNA damage caused by radiation is life threatening to a cell.

1.3.1 Radiation can create double-strand breaks.

DNA is the blueprint for life; it encodes all the necessary factors to produce a functionally operational cell, and its maintenance is critical not only for viability of the cell but long term survival of the species. Damage to a cell's DNA often has harmful consequences, including mutagenesis or cell death, if left unrepaired[36]. DNA injuries can result from either endogenous or environmental sources[37]. The former refers to damage that naturally occurs within the cell and is often a normal byproduct of life. One such example is the production of reactive oxygen species (ROS), which may leak from the electron transport chain or be generated as intermediates of metabolic processes. ROS are highly electrophilic and can readily remove hydrogens from the DNA backbone or add themselves to nitrogenous bases[37,38]. Environmental damage includes insults that come from physical or chemical sources and originate outside the cell. One important mode of environmental damage is radiation, which can be used to study DNA

repair mechanisms. Different forms of radiation vary in the level of energy they carry; two of these are ultraviolet (UV) radiation or ionizing radiation (IR), which can result in distinct but numerous lesions including dimerization of the nitrogenous bases, production of ROS, and DNA strand breaks[37-39].

Ultraviolet radiation is one of the most studied forms of DNA damage because it is biologically relevant and because UV sources are readily available and easy to use in a common laboratory setting[37]. The UV spectrum can be subdivided according to wavelength: UV-A (320-400 nm), UV-B (295-320 nm), and UV-C (100-295 nm). Solar radiation that reaches biological life is composed of UV-A and UV-B, while UV-C is typically absorbed in the atmosphere[37]. Laboratory studies most often utilize UV-C light which emits 254 nm, close to the peak absorbance of DNA at 260 nm and thus fairly specific for DNA lesions. Although UV-C is not as biologically relevant as other UV wavelengths, studies have demonstrated that the major lesions produced from UV-B light appear in proportional amounts from UV-C exposure[40]. Therefore, the repair of lesions produced UV-C does indeed have biological relevance. These lesions consist primarily of cyclobutadipyrimidine dimers (i.e. thymine cyclobutane dimers, commonly referred to as thymine dimers) and secondarily as pyrimidine (6-4) pyrimidone dimers[41]. UV-C light has also been demonstrated to inflict DNA with single-strand breaks (SSBs), although it is not typically considered a direct source of double-strand breaks (DSBs)[42]. However, it has been observed that DSBs can be indirectly produced in the cell after UV irradiation[43,44]. These may be due to encounters of replication machinery with SSBs that result in DSBs. Such a situation is termed a *collapsed* replication fork[37,45]. Replication machinery is also hypothesized to stall upon reaching and unrepaired dimer, nick one DNA strand, and resume replication elsewhere, leaving regions of single-stranded DNA (ssDNA)[46-48].

Ionizing radiation is a second important physical source of DNA damage. It has been calculated that one photon of ^{60}Co radiation, a form of gamma radiation, can produce 36,000 hydroxyl radicals within the cell, which can result in a wide variety of lesions affecting both DNA as well as other cellular components[38]. Injuries to DNA can be either direct or indirect. Direct damage occurs when the DNA

molecule itself absorbs energy from the radiation, and the molecule itself becomes ionized. Indirect damage results from the ionization of species surrounding the DNA, such as water, which then attack it[37]. Most of the damage suffered from ^{60}Co radiation is indirect and consists of multiple DNA lesions, two of which are DNA SSBs and DSBs[37,38]. A study of damage on eukaryotic cells demonstrated that relatively low dose of 100 rads of X ray radiation (another form of IR) produces approximately 40 DSBs and 1000 SSBs per cell[49]. Within this study it was also emphasized that statistically a cell likely does not survive due to avoidance of the radiation; instead, functional and efficient mechanisms of recognizing and repairing breaks likely combat damage.

Double-strand breaks (DSBs) are one of the most dangerous lesions that threaten DNA. Such lesions are inherently problematic due to the potential separation of the broken ends. DSBs can either occur accidentally (i.e. unintentionally) within the cell or they can be deliberately created via endogenous pathways such as V(D)J recombination and meiotic homologous recombination for the specific purpose of promoting genetic diversity. In either case, immediate repair is critical, as unrepaired DSBs can destabilize the genome or lead to cell death.

1.3.2 There are two major mechanisms for repairing cellular DSBs.

Two predominant mechanisms that cells utilize to repair endogenous DSBs are HR and nonhomologous end-joining (NHEJ). Currently, it appears that all known organisms have HR mechanisms, however NHEJ has not yet been detected in some bacteria and the domain *Archaea*. These two pathways have distinctly different mechanisms. HR is a relatively complicated pathway that utilizes many proteins factors and a homologous template to restore DNA to its identical state prior to when damage occurred. NHEJ requires fewer protein factors and fewer steps to repair broken DNA but can lead to mutations in the original DNA sequence because it does not utilize template DNA. It is generally believed that HR is utilized

in replicating cells that have an identical copy of DNA available and in close proximity, whereas NHEJ occurs predominantly in cells in G1 or resting phase that do not have readily available template DNA[50].

1.4 Homologous recombination is a pathway for repairing DSBs and other DNA lesions

1.4.1 Overview of homologous recombination.

Homologous recombination is a pathway that repairs DNA strand breaks and is common to all domains of life[51,52]. HR is classically thought of as a DSB repair pathway but it can also repair SSBs, interstrand crosslinks, and single-strand gaps[45]. One common use of HR is to fix errors encountered in replication. It has been suggested that the majority of unintentional DSBs encountered within the typical cell are not due to environmental sources but instead from replication machinery encountering nicks created by endogenous sources such as reactive oxygen species[53]. HR may also be commonly employed to repair gaps created by collapsed replication forks, which occur in an estimated 18% of cells undergoing a single round of replication[53,54].

HR in all known organisms proceeds through the same basic steps: presynapsis, synapsis, and postsynapsis (Figure 1.4). In classical DSB repair, detection of a break is followed by processing of the broken ends by nucleases to expose 3' overhangs. Presynapsis also includes multimerization of the HR strand exchange protein on resected ssDNA to form a presynaptic filament[45]. Synapsis involves a protein-mediated homology search for an exposed strand's complement and the invasion of the presynaptic filament into the duplex homologue. In this step, strand exchange and complementary pairing occur, resulting in the displacement of the non-paired duplex strand to form a structure known as a displacement loop (D-loop). The D-loop DNA is thus available to anneal with its homologue, the other resected 3' end in a process known as second end capture[45]. Branch migration then occurs to extend the heteroduplex molecule, called a Holliday junction (HJ) to maximize pairing between the single strand and its homologue. In postsynapsis, a polymerase synthesizes new DNA to restore missing nucleotides, resulting in error free

repair because homologous DNA is used as a template. The Holliday junction will finally be cleaved to result in two double-stranded DNA homologues, each with a nick that is sealed by a DNA ligase[55].

1.4.2 Homologous recombination in bacteria.

Eubacteria are distinct from eukaryotes in that they are haploid the majority of their life cycle, except during bacterial replication for the purpose of division (reviewed in[56]). Homologous DNA can be provided by introducing exogenous DNA via transformation, conjugation, or viral transduction; it also can be provided through replicated daughter DNA. The former events are likely not recurrent in the normal bacterial lifecycle, thus it has been proposed that the major purpose of HR in eubacteria is to repair stalled replication forks, which occur more frequently[53].

The central steps of eubacterial HR are catalyzed by RecA (Table 1.1). This multifunctional protein performs homology search, strand invasion, and strand exchange in the presynaptic and synaptic steps of HR[55-57]. It is aided by a tetrameric single stranded DNA binding protein, SSB, which assists RecA in presynapsis by removing secondary structure from ssDNA to allow RecA to then load[58,59]. RecA is a ssDNA-dependent ATPase that forms right handed helical filaments around exposed ssDNA and dsDNA[60,61]. The binding of ATP stabilizes RecA-RecA interactions to allow polymerization along ssDNA[62]. Crystal structures of the RecA-ssDNA filament reveal that each monomer binds three nucleotides of ssDNA and hold it in a B-like structure, similar to duplex DNA[62].

Extensive study of bacterial HR has revealed two distinct mechanisms of presynapsis. Interestingly, these correlate to the type of substrate. The first employs the RecBCD complex, which repairs linear dsDNA ends that come from DSBs caused by ionizing radiation damage, from the entry of exogenous DNA via viral transduction or conjugation, or from breaks produced when other repair mechanisms are lacking[63]. The second method employs the RecFOR complex. This mechanism is responsible for repairing gaps in DNA resulting from sources such as collapsed replication forks (reviewed

in[56]). The RecFOR pathway is thought to be the primary HR mechanism responsible for the repair of gaps resulting from UV-induced lesions which have not been repaired via nucleotide excision repair (NER)[63,64]. Because gapped DNA is more likely to be encountered than DSBs, the RecFOR pathway is likely to be used more often than the RecBCD pathway.

The RecBCD complex has helicase activity, which preferentially unwinds dsDNA ends, and endonuclease activity, which cleaves duplex DNA and resects it to allow for 3' end invasion of the homologous template[65,66]. Specifically, when RecBCD detects a DNA end, it travels along it, cutting the dsDNA until it reaches a recombination hotspot known as the Chi (χ) hotspot. The complex creates a nick at χ, which alters its nuclease activity so that it cuts only one strand of the duplex, leaving an exposed ssDNA end[67,68]. After nicking DNA at χ, RecBCD also assists RecA loading onto the exposed 3' end[69].

The RecFOR pathway works in lieu of the RecBCD pathway when the HR substrate is gapped DNA. This pathway is initiated with a helicase, RecQ, which can open dsDNA; the exposed ssDNA is then bound by SSB to inhibit re-annealing[70]. RecJ, a nuclease with preference for 5' ssDNA ends, can also further expose ssDNA to act as a substrate for strand invasion[71]. The RecFOR complex then assists in loading RecA onto ssDNA to make a protein-DNA filament. This complex specifically has the ability to load RecA onto regions of gapped DNA and can additionally stimulate RecA strand exchange activity in the presence of SSB[72,73].

Once RecA has been loaded onto ssDNA, the protein filament searches for homologous DNA and then invades duplex DNA[55]. The search for and pairing with homologous DNA is believed to be largely due to Watson-Crick interactions. The B-like structure of the ssDNA maintained due to interactions with the RecA filament both encourages these interactions and inhibits non-Watson-Crick pairing[74]. RecA then catalyzes strand exchange, in which the invading strand pairs with its complement[57]. The displaced ssDNA is stabilized by SSB and can then pair with its respective homologue[75].

In postsynapsis, interactions between homologues are maximized by branch migration, which is catalyzed by the RuvAB proteins in an ATP dependent manner[76,77]. RuvA is hypothesized to target Holliday junction structures and RuvB is believed to be the motor behind the branch migration itself[76,77]. After DNA Polymerase I synthesizes new DNA to restore missing information, RuvC endonuclease cleaves the HJs and a DNA ligase seals the fully restored DNA duplexes[55,78].

1.4.3 Homologous recombination in eukarya

Homologous recombination is, in general, more complex in eukaryotes than it is in bacteria. Specifically, it utilizes more accessory factors to carry out the basic steps. The pathway is conserved from yeast to humans, however HR in yeast is slightly more streamlined and requires fewer proteins than humans, thus HR in *Saccharomyces cerevisiae* will be the primary eukaryotic model considered here[79]. Recombination in eukaryotes has an additional function because it serves to both repair damaged DNA and mediate the crossing-over of chromosomes during meiosis[79]. The meiotic HR pathway is analogous to the damage repair pathway and is in general mediated by either homologues or the very same proteins. The key proteins that mediate mitotic HR in eukaryotes are members of the Rad52 epistasis group. The genes that comprise this group in *S. cerevisiae* are *mre11, rad50, xrs2, rfa1, rad51, rad52, rad54, rad55, rad57,* and *rad59.* These genes were identified by the sensitivity of mutants to ionizing radiation[79,80].

Once a DNA strand break has been created, the Mre11/Rad50/Xrs2 (MRX) complex binds the ends and assists in processing. Mre11, a nuclease, and Rad50, an ATPase, can bind each other in the absence of Xrs2 and are conserved in all domains of life[16,81]. The involvement of Mre11 nuclease activity in HR is debated because mutants with abolished nuclease activity survive ionizing radiation nearly as well as wild type cells[82]. Furthermore, Mre11 is a 3' – 5' exonuclease, and resection of DSBs *in vivo* would presumably require a 5' – 3' exonuclease activity to expose a 3' ssDNA region[83]. However, recent evidence strongly supports a role for Mre11 endonuclease but not exonuclease activity in the processing of

DSBs[84]. The MRX complex does appear to have an additional structural role as it may facilitate tethering of broken ends so that they do not become lost before repair can occur[82,85]. Homologues of yeast Xrs2, which are thought to function as a checkpoint mediators, are only found in eukaryotes and bear more functional than sequence similarity[85]. The nuclease responsible for resection of DNA ends to reveal a 3' ssDNA tail is not clear in eukaryotes. In yeast, one candidate is Exo1, a nuclease with 5' to 3' exonuclease and flap endonucleases activity[86]. Exo1 mutants in yeast experience impaired recombination, however it is not a member of the *rad52* epistasis group[87].

Exposed 3' ssDNA tails are bound by replication protein A (RPA), a heterotrimer that is encoded by three separate genes, including *rfa1,* which are all essential for cell viability[80,88]. Like bacterial SSB, RPA stimulates the formation of the presynaptic filament by removing secondary structure from ssDNA. It also greatly stimulates the strand exchange activity of Rad51, the major eukaryotic strand exchange mediator[89]. After strand exchange has occurred, RPA has a postsynaptic role in binding displaced DNA[90]. Rad51 is a DNA dependent ATPase whose activity is stimulated by ssDNA[91]. Rad51 forms right handed helical filaments on broken DNA ends and then catalyzes strand exchange between homologous DNA strands[61,92].

In vitro, RPA must be added to strand exchange reactions after Rad51 has been added or else it immediately binds ssDNA and inhibits Rad51 filament formation and strand exchange[93]. However, in the cell RPA is constitutively available, thus requiring the assistance of multiple accessory proteins. Furthermore, Rad51 alone has very little strand exchange activity and these accessory proteins help stimulate this activity. Key accessory factors that help mediate this process in yeast include Rad52, Rad59, Rad55, Rad57, and Rad54.

Rad52 is a multimeric, ring-shaped protein that interacts with Rad51 *in vivo* and stimulates the strand exchange activity of Rad51 in the presence of RPA[94,95]. Rad52 is critical for Rad51 filament formation; it is hypothesized to be recruited to ssDNA by RPA where it then assists Rad51 in displacing RPA[92,94,96]. It also has the ability to anneal short stretches of homologous DNA and may be involved in the

homologous pairing step of HR[94,97]. Rad59 is homologous to the amino end of Rad52 and has some similar *in vitro* activities, such as the ability to bind DNA and promote annealing of short homologous sequences[97]. The two proteins also interact *in vivo* and are hypothesized to work together to mediate presynapsis[97-99]. Rad55 and Rad57 are APTases and Rad51 paralogues[100,101]. Rad55 and Rad57 are believed to form a stable complex in the cell, where they are hypothesized to play a role in assisting Rad51 filament formation and strand pairing/exchange; indeed, the heterodimer has been demonstrated to stimulate Rad51 strand exchange in the presence of RPA[92,100,102].

The final key accessory factor in mitotic HR is Rad54, a dsDNA-dependent ATPase that directly interacts with Rad51[103-105]. *In vitro* studies of Rad54 from yeast and humans have revealed multiple activities, leading to hypotheses of its involvement in multiple steps of HR. Initially, Rad54 may have a presynaptic role remodeling chromatin because it has been found to reposition nucleosomes on dsDNA[106]. This activity is stimulated by Rad51/ssDNA filaments and can occur in the absence of homologous DNA, indicating that Rad54 may assist in repositioning nucleosomes during homology search[106]. Rad54 can additionally bind Rad51/dsDNA and Rad51/ssDNA complexes and, like other accessory factors, stimulate Rad51-mediated strand exchange[106]. The mechanism of stimulation appears to be through binding Rad51/DNA presynaptic filaments and minimizing the dissociation of Rad51 from DNA[106]. Paradoxically, Rad54 has also been demonstrated to dissociate Rad51/dsDNA filaments[105]. *In vivo,* Rad51 may stably reside on homologous dsDNA after strand exchange has occurred. Alternatively, Rad51 or other interfering proteins may be initially bound to homologous template. In either case, Rad54 may "clear the way" by removing interfering proteins in order to allow HR to proceed[105]. Rad54 has a strong affinity for D-loop structures, and it promotes D-loop formation in the presence of Rad51 and RPA[103,107]. Finally, Rad54 may facilitate postsynapsis by catalyzing branch migration of Holliday junctions[107]. While the broad spectrum of *in vitro* activities has implications on Rad54's *in vivo* roles in HR, the exact roles of Rad54 *in vivo* remain to be fully elucidated.

The resolution of Holliday junctions has remained an elusive concluding step of HR in eukaryotes. This is in part due to the fact that HJs can be resolved in multiple ways and that some of the proteins with resolvase activity are thought to perform resolution as a secondary rather than primary function; the HJ resolvase was also believed to be present in low numbers within the cell[108]. Factors that may play a role in *S. cerevisiae* HJ resolution include Yen1, Mus81/Mms4, and Sgs1/Top3. Human GEN1 and its yeast homologue, Yen 1, have been recently identified as eukaryotic proteins responsible for HJ resolution[108]. These proteins were identified independent of each other from resolvase activity assays in human and yeast cells and subsequently found to be homologues. Their primary substrate specificity is for four-way HJs, and cleaved junctions can then be religated *in vitro*. Yen1 and GEN1 belong to the Rad2/XPG family of nucleases and are not homologous to bacterial or archaeal HJ resolvases[108]. Endogenous Mus81-Eme1 in *Schizosaccharomyces pombe* has a high substrate specificity for nicked HJ-like structures and an ability to nick continuous structures as well[109]. Furthermore, Holliday Junctions have been found to accumulate in *pol1mus81* mutants; DNA polymerase and Mus81 act synergistically and the *pol1* mutation allows HJ accumulation to be seen[109]. The role of Mus81/Mms4, the *S. cerevisiae* homologue of Mus81/Eme1, is less clear, however, as it does not have high substrate specificity for Holliday Junctions, although the complex does interact with the Rad54 protein[108]. Sgs1 and Top3 have also been proposed to be involved in the resolution step of HR[45,110]. Sgs1 is a member of the RecQ helicases and several of its homologues, including RecQ in *E. coli* and Wrn in humans, have been proposed to be involved in HJ resolution[111]. Top3 is a type 1 topoisomerase that could open DNA strands to allow dissociation of the duplex via the Sgs helicase[112]. While progress has been made in this area, much remains to be done to clarify the mediators involved in eukaryotic HR resolution.

1.4.4 Homologous recombination in archaea

Homologous recombination occurs in all domains of life, however it is least well characterized in archaea due in part to the relatively late realization of their distinctness from eubacteria. Recombination in archaea has been a point of interest for some time because many of its members are expected to contend with continual DNA damage due to living in harsh conditions, such as high temperature and acidic pH[14]. Aside from damage repair, endogenous uses of HR by archaea are similar to bacterial uses, including incorporation of foreign DNA via viral transduction, conjugation, transformation, or reinsertion of chromosomal DNA from insertion element hopping[14].

Studies of HR proteins in archaea have revealed a pathway resembling the eukaryotic one because many archaeal recombination proteins have higher homology to their eukaryotic counterparts than their prokaryotic ones[14,52]. Archaea have Mre11 and Rad50 homologues that are believed to play a role in processing breaks, however they lack Xrs2 homologues[113]. Crystal structures of the Mre11/Rad50 tetramer have given key insights into understanding the structure and function of these proteins in archaeal and eukaryotic systems[16]. In archaea, Mre11 and Rad50 are encoded in the same operon, which also encodes two other genes, NusA and HerA, in most species[114]. NusA is a 5'-3' nuclease and HerA is a bidirectional helicase; the two have been demonstrated to directly interact via gel filtration[114-116]. Like bacteria, archaea can coordinate the transcription of genes by arranging them in operons, and genes contained within operons are often involved in similar pathways. Thus, it has been hypothesized that NusA and HerA may play a role in archaeal HR together with Mre11/Rad50[114]. *In vitro* analysis of these four proteins has revealed that together they are capable of resecting the 5' strand of duplex DNA to reveal 3' ssDNA[116]. While the catalytic ability of all four proteins was required for maximal resection, the nuclease activity of Mre11 was partially dispensable, allowing for a product to be formed, albeit less efficiently[116]. Consistent with emerging eukaryotic models, endonucleolytic cleavage mediated by Mre11 was observed that allowed the production of short 3' ssDNA ends[116]. Finally, in the presence of the archaeal strand

exchange protein RadA, Mre11, Rad50, HerA, and NusA together were found to promote optimal strand invasion of supercoiled dsDNA to produce joint molecules[116]. More studies are needed to fully elucidate the role of these proteins in HR, but current data for their involvement is promising.

Both crenarchaea and euryarchaea utilize ssDNA binding proteins to sequester and/or stabilize ssDNA, however the structure of these proteins differs between the two phyla and between species. The first proteins to be identified were in euryarchaea and found to bear much more homology to eukaryotic RPA than bacterial SSB[117]. Euryarchaeal RPA is typically encoded on one to three different genes; the mono- or multimeric protein usually contains four DNA binding domains, similar to the bacterial and eukaryotic ssDNA binding proteins[117]. Like in bacterial and eukaryotic systems, euryarchaeal RPA has been found to interact with RadA *in vivo* and to stimulate strand exchange[118]. The ssDNA binding protein in crenarchaea has higher homology to RPA than SSB, but, like bacterial SSB, it is encoded by a single gene and the resultant protein can be tetrameric[119]. Thus, the crenarchaeal ssDNA binding protein resembles SSB structurally. Crenarchaeal SSB has also been found to stimulate both *E.coli* RecA mediated strand exchange and SsoRadA-mediated strand exchange[119,120]. The full extent of SSB's role HR in archaea is under debate. In bacteria and eukaryotes, one important role of ssDNA binding proteins is likely to inhibit secondary structure formation of resected DNA during presynapsis, however many archaea live at high temperatures that would likely melt secondary structure[14,59,89]. One hypothesis is that SSB may protect ssDNA from heat an acid degradation in hyperthermophilic species. Nonetheless, learning what functions this protein plays in an environment absent of significant secondary ssDNA structure could give a better understanding of additional roles SSB and RPA in bacterial and eukaryotic HR, respectively.

The major strand exchange protein in archaea is RadA, which has been found to bear more similarity to eukaryotic Rad51 than bacterial RecA[121]. RadA has been purified and characterized from a variety of species and, like RecA and Rad51, forms a right handed, helical filament around DNA[52]. In

addition, it is a DNA dependent ATPase and can promote strand exchange and D-loop formation[122,123]. The full length protein has also been crystallized, which has led to insights into archaeal, bacterial, and eukaryotic homologues[17]. As previously mentioned, paralogues of Rad51 (i.e. Rad55 and Rad57) are utilized in eukaryotic HR to assist Rad51. Likewise, RadA paralogues have been found in archaea, although their roles are poorly understood[52]. These paralogues differ between the euryarchaeal and crenarchaeal phyla. Euryarchaea utilize RadB, a protein that is smaller than RadA and bears 38-54% similarity to yeast Rad51[52]. Compared to RadA, RadB has a lower ATPase activity and binds ssDNA and dsDNA with higher affinity[124]. Direct interactions between RadB and RadA are equivocal, however a direct interaction between RadB and the archaeal HR resolvase Hjc has been demonstrated[124]. RadB's role is elusive because, in contrast to the eukaryotic Rad51 paralogues, RadB has not yet been demonstrated to stimulate D-loop formation or RadA-mediated strand exchange activity, however it does appear to alter the nuclease activity of Hjc and may play a role in postsynapsis alongside this protein[124].

Multiple RadA paralogues within single species have been found in crenarchaea and have been tentatively named aRadC (short for archaeal RadC) proteins[52,125]. Largely uncharacterized, these proteins are distinct from the euryarchaeal RadB and more similar to bacterial RecA than Rad51, although the similarity is small (25-27%)[52]. Of the three paralogues found in *S. solfataricus*, *in vitro* studies of one, the protein product encoded by SSO2452, demonstrated an ATPase activity similar to that of RadA which is highly stimulated by ssDNA and dsDNA[126]. This paralogue was also capable of catalyzing stand exchange between short oligomeric substrates, however it was not found to catalyze D-loop formation with longer substrates or stimulate RadA-mediated D loop formation when the two were incubated together in the presence or absence of SSB[126]. Due to its higher affinity for binding DNA, it was hypothesized that this aRadC may sequester DNA from RadA and thus obstruct D loop formation and strand exchange[126]. As with RadB, more studies are necessary to determine if these paralogues truly play a role in archaeal HR and to define what their functions are.

A putative Rad54 homologue was first reported in the crenarchaeote *S. solfataricus* in 2001[52]. Since then, the catalytic domain of this protein has been crystallized and *in vitro* studies reveal its true identity as a Rad54 homologue[35,127]. Like eukaryotic Rad54 proteins, SsoRad54 has dsDNA stimulated ATPase activity, can alter the topology of dsDNA by inducing supercoiling, and directly interacts with RadA[35]. Furthermore, it lacks strand exchange activity itself but promotes RadA-mediated strand exchange activity. Other Rad54 homologues have not been reported in bacteria or other archaea, however this may be due to sequence divergence rather than complete absence in these organisms[35]. BLAST searches for bacterial homologues of SsoRad54 reveal up to 39% identity and 59% similarity to bacterial HepA, a DNA-binding ATPase known for interacting RNA polymase[35]. It is unknown if HepA plays a role in bacterial HR, but the high homology of HepA to SsoRad54 suggests it may be involved.

Archaeal HJ resolvases have been discovered and characterized in both crenarchaea and euryarchaea[128-130]. The homologues are called Hjc (short for Holliday Junction Cleavage) proteins and bear approximately 30% or more sequence identity to each other, although they are not homologous to known bacterial or eukaryotic resolvases[128,129]. Hjc efficiently cleaves four way junctions and this activity may be sequence specific[128,129]. Hjc forms a dimer like other resolvases and can resolve recombination intermediates created *in vitro* by RecA[128]. Evidence of a second endonuclease, named Hje (short for Holliday junction endonucleases), in *S. solfataricus* has been reported from fractionated whole cell extracts. Hje can cleave four way junctions and three way junctions containing an unpaired bulge in a sequence independent manner[130].

Current understanding of archaeal HR lags behind what is known in eukaryotic and bacterial HR because of the relatively late separation of these organisms into a unique domain of life and consequently delayed study of their HR pathway. The emerging picture reveals some unique features embedded in a generally eukaryotic-like pathway. While a much is known about eukaryotic HR, studies have been limited by the difficulty of purifying involved proteins and understanding their contributions. Due to their stability

and robust nature, archaeal homologues have proven themselves to often be much easier to work with. Thus, as details of the structures and mechanisms of archaeal HR become elucidated, insights will be gained into eukaryotic systems as well.

1.5 Nonhomologous end-joining is a second method of repairing cellular DNA DSBs.

1.5.1 Overview of nonhomologous end-joining

The second well characterized pathway of DSB repair is the nonhomologous end-joining pathway. This pathway does not rely on a homologous template to mend broken DNA, but instead likely uses protein factors to bridge together broken ends so that they can be maintained in close proximity and then repaired[131,132]. Thus, NHEJ is believed to be favored during periods of the cell cycle, such as G0 and G1 phase, in which a sister chromatid or newly replicated chromosome is not available as a template for use in the HR pathway[133]. Because the majority of DSBs created within the cell are not blunt ended, NHEJ repair is likely mediated through small regions of complementarity between broken ends[133]. NHEJ is not commonly known for repairing breaks other than DNA DSBs, unlike HR which can act on other substrates such as collapsed replication forks. However, in addition to repairing unintended DSBs, NHEJ in higher eukaryotes has an additional role in joining together variable (V) diversity (D) and joining (J) gene segments in a process known as V(D)J recombination[134]. V(D)J recombination is a highly important process creates a diverse repertoire of B-cell and T-cell receptors that allow the immune system to detect a vast array of immunogens and thus protect it from infection.

Depending on the nature of the broken DNA ends, NHEJ can proceed through one of two similar mechanisms (Figure 1.5). The first is basic NHEJ, which requires the simple ligation of compatible DNA ends. This pathway involves binding the DNA ends, bringing them together (synapsis), and ligation. Broken DNA ends with regions of noncomplementarity or nucleotide loss necessitate processive NHEJ.

Here, end-binding and synapsis occur as before, however nucleases and/or polymerases are utilized to either cleave off overhanging flaps or fill in gaps prior to final ligation of the broken ends.

1.5.2 Bacterial nonhomologous end-joining

NHEJ was discovered in bacteria after it had already been found in eukaryotes. This discovery resulted from the identification of Ku and ATP-dependent DNA ligase (ADDL) homologues (Table 1.2)[135-137]. Interestingly, not all bacteria have apparent NHEJ mechanisms, and there is currently no known pattern of identifying which members of a phylum or class will utilize it and which will not[50]. However, NHEJ is found in many bacterial species whose primary existence is under stringent growth conditions or stationary phase, such as *Bacillus*, *Clostridium*, and *Pseudomonas* species[138].

Ku and an ADDL are the two core components of bacterial NHEJ. The bacterial "Ku core" is small and forms a homodimer in contrast to the Ku heterodimer found in eukaryotes[139]. Bacterial NHEJ ligases are often located in the same operon as Ku and are can be multifunctional, including nuclease and polymerase domains which allow processing capabilities without additional protein factors[135,140,141]. Thus, the two component bacterial NHEJ pathway is a very simple method of repairing double-strand breaks.

Genetic studies of bacterial NHEJ components have been done primarily in *Bacillus* and *Mycobacterium* species. In *B. subtilis*, *YkoV* (Ku) and *YkoVYkoU* (Ku/ADDL) mutants are more sensitive to ionizing radiation than wild type cells, and are more sensitive to spore forming conditions, such as dry heat[142]. Transcriptome analysis has revealed that this operon is under the control of σ^G, a regulatory protein in spore formation[143]. Thus, NHEJ appears to be used primarily when *B. subtilis* exists as an endospore. Ku and ligase deficient strains of *M. smegmatis* are sensitive to IR only in stationary phase[144,145]. Moreover, HR deficient *recA* mutants are as sensitive to IR as *recA/ku/ligD* mutants in logarithmic growth phase, but the latter are 1000 fold more sensitive to IR in stationary phase cultures.

Overall, evidence suggests that NHEJ is utilized in stress-related conditions, although the interplay between this pathway and HR is not yet fully understood[144].

1.5.3 Eukaryotic nonhomologous end-joining.

NHEJ was first discovered and remains best characterized in eukaryotes (reviewed in [138,146]). This pathway has been characterized in both mammals and yeast[134,147]. The two pathways are similar and utilize many homologous proteins, however, like yeast HR, yeast NHEJ is simpler than mammalian NHEJ and the proteins involved in the *S. cerevisiae* pathway will be considered here. It should be noted, however, that a few differences are known between yeast and mammalian NHEJ. Two of these include the use of the MRX complex in yeast NHEJ but not apparently in the mammalian pathway, and the use of the DNA dependent protein kinase catalytic subunit (DNA PK_{cs}), a kinase absent in yeast but utilized in mammalian NHEJ. DNA PK_{cs} plays an important role in mammalian NHEJ; it is a kinase that binds broken DNA ends along with Ku and appears to play a role in tethering broken DNA ends[148,149].

In the *S. cerevisiae* NHEJ pathway, creation of a DNA DSB is followed by the immediate binding of the Ku70/80 heterodimer and MRX complex to the broken DNA[150]. The Ku70/80 heterodimer is a DNA end-binding protein that serves as a hallmark protein for the identification of NHEJ pathways[151]. The crystal structure of the mammalian Ku70/80 heterodimer revealed first, that it forms a ring-like structure, and second, that the residues on the inside of the ring are polar[146]. This structural information elucidated the heterodimer's affinity for binding DNA ends[152] . In NHEJ, Ku proteins appear to have a dual role in recruiting other factors, such as Dnl4, the DNA ligase responsible for the final ligation step, to broken ends and potentially tethering DNA ends together to allow for synapsis to occur[150,153,154]. Eukaryotic Ku has additional functions within the cell, such as maintenance of telomere length and transcriptional silencing of telomeres[147,155,156]. Such activities complicate interpretations of *in vitro* findings due to the seemingly contradictory activities revealed in this protein: Ku is involved in both joining broken DNA ends within the

chromosome and in guarding linear telomeres which should *not* be joined. Mutational analysis of *S. cerevisiae* Ku70 has implicated involvement of the final thirty C-terminal residues of this protein in DNA binding, protection of telomere length, and NHEJ; the residues closest to the C-terminal appear to be specifically involved in telomeres maintenance but dispensable for NHEJ activity[157].

Evidence for the involvement of the MRX complex in NHEJ has come through mutation studies which revealed that mutants of each member of the complex have reduced end-joining activities similar those of Ku mutants[156]. Furthermore, similar loss of NHEJ was observed in double mutants containing mutations of the yku70 gene and a member of the MRX complex, indicating that these factors are epistatic. The exact role of MRX is not clear, however it does not appear to involve the nuclease activity of Mre11[150,158]. Recent studies indicate that the MRX complex arrives at broken ends immediately following the creation of DSBs and appears to function in the release of Ku and Dnl4 from DNA, in a manner that is dependent on the ATPase activity of Rad50[150,159]. Like Ku, the MRX complex is active in other cellular pathways, such as HR (as described earlier) and telomere maintenance, which make it difficult to clarify its role in NHEJ[156].

If the broken DNA ends are compatible, the break can be rejoined through a simple ligation step. Ligation of DNA ends is mediated by an ATP-dependent Ligase IV homologue encoded by the *dnl4* gene in *S. cerevisiae*[160]. Dnl4 protein interacts with Lif1, a protein that stabilizes it and stimulates the Dnl4-mediated ligation of DNA ends[161]. Lif1 may also play a role in directing Dnl4 to DSBs already bound by Ku[162]. Kinetic studies reveal that Dnl4 arrives at DSBs after the Ku heterodimer and MRX complex[150]. Indeed, Dnl4/Lif1 binding of DNA ends is Ku dependent; the complex also serves to stabilize the binding of Ku to DNA ends[159]. The final protein known to be involved in the non-processive *S. cerevisiae* NHEJ pathway is Nej1, which can bind Lif1 but not Dnl4[163,164]. Nej1 additionally can bind DNA, however its role in NHEJ remains unclear[165].

If broken ends are not compatible, processing is necessary for ligation to occur. Identified processing factors in *S. cerevisiae* include the Pol4 polymerase, a member of the PolX family of polymerases, and the Rad27 nuclease, a homologue of the mammalian FEN-1 flap endonuclease[166,167]. Specifically, Pol4 is hypothesized to fill in gapped regions of DNA and Rad27 is believed to cleave overhanging noncomplementary flaps[168,169]. Both of these proteins appear to interact with Dnl4 at the site of the DSB[168,169]. In a proposed model, these three proteins coordinate in action to mend broken, incompatible DNA ends: Pol4 initially fills in gaps, Rad27 endonuclease then cleaves flaps, and Dnl4 aided by Lif1 and Nej1 finally seals the nicked DNA[169].

1.5.4 Nonhomologous end-joining has not been reported in archaea.

In 2001, two reports came out which identified a putative Ku core located adjacent to a ligase homologue in the archaeon *Achaeoglobus fulgidus*[135,136]. Since that time, no experimental evidence has confirmed the identity of the putative Ku or the presence of end-joining activity in any archaeon. Nonetheless, these findings hint that NHEJ may be a pathway common to all three domains of life. Archaea also encode genes for Mre11 and Rad50, which function in yeast NHEJ[170]. A potentially quick and efficient end-joining pathway could be very useful to archaea, some members of which grow in harsh environments, such as temperatures exceeding 100°C, that would likely yield constant DSBs[171]. Furthermore, as *Sulfolobus* is haploid except during cell replication and division, NHEJ would allow the repair of breaks in G1 phase when homologous DNA is unavailable.

1.6 Purpose of this work

The overall goal of my research in the Haseltine lab has been to investigate and understand DNA double-strand break repair in the archaeon *Sulfolobus solfataricus* for the purpose of utilizing the repair pathways of this model system to better understand repair in eukaryotes. Specifically, my research

involved two different pathways of double-strand break repair. First, I studied the role of Rad54 in damage repair following UV irradiation. The work described in Chapter 2 demonstrates that archaeal HR is likely involved in the recovery of *S. solfataricus* from UV damage. It also shows, for the first time, upregulation of Rad54 transcripts in response to UV damage in an archaeon. This work describes strain dependent differences in damage recovery and reveals natural strain dependent differences in the Rad54 gene itself. Here, I also characterized for the first time the response of *S. solfataricus* to ionizing radiation. In Chapter 3 I investigate an alternate method of DSB repair. The work detailed in this chapter describes preliminary efforts I performed to characterize a previously unidentified end-joining pathway in archaea. A potential archaeal Ku homologue was identified in *S. solfataricus* and results indicate that transcripts of this gene are upregulated in response to DNA damage. Nonhomologous end-joining is an alternate DSB repair pathway that has been characterized in eukaryotes and some eubacteria. In this section I present an *in vitro* assay that I modified for use in *S. solfataricus* and show preliminary evidence for the presence of an end-joining pathway in the third domain of life.

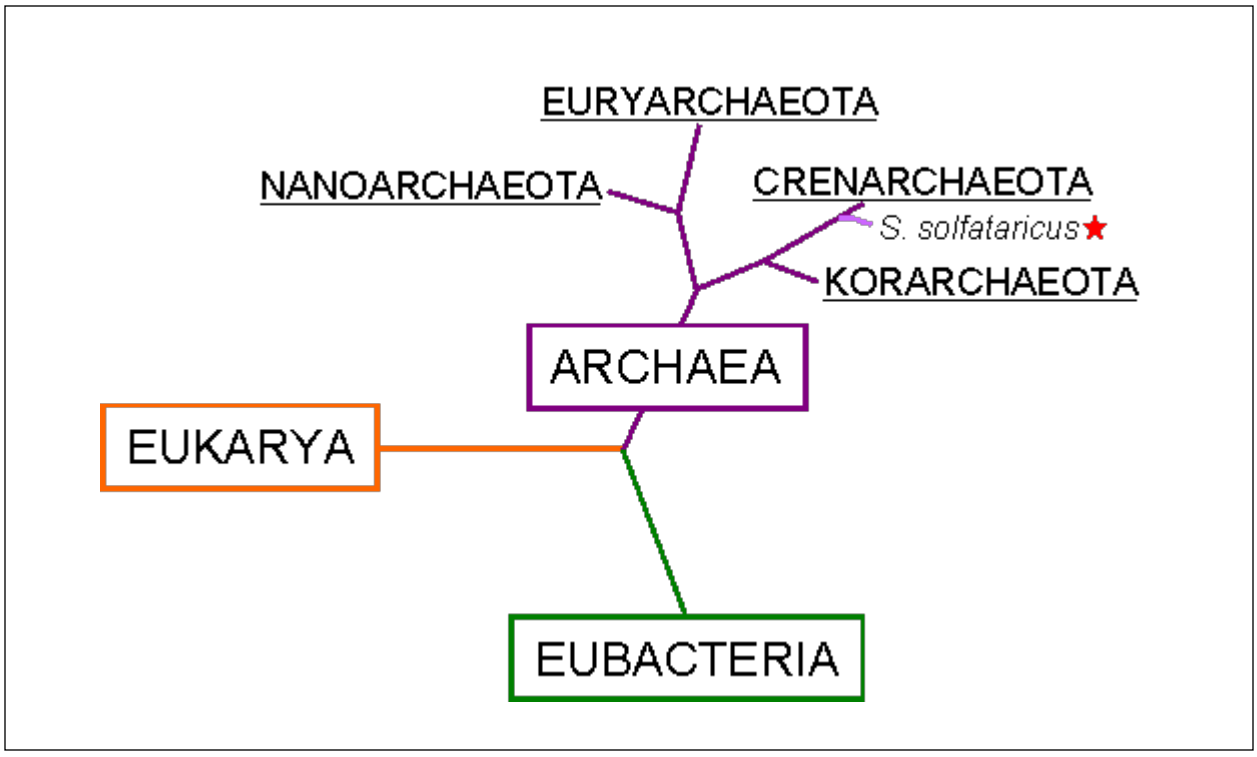

Figure 1.1 Unrooted phylogenetic tree of life (adapted from[172,173]). *Archaea* were separated into a separated into a separate domain of life distinct from eubacteria and eukaryotes in 1990. *Sulfolobus solfataricus* (red star) is a thermoacidophilic microorganism and member of the phylum *Crenarchaeota*.

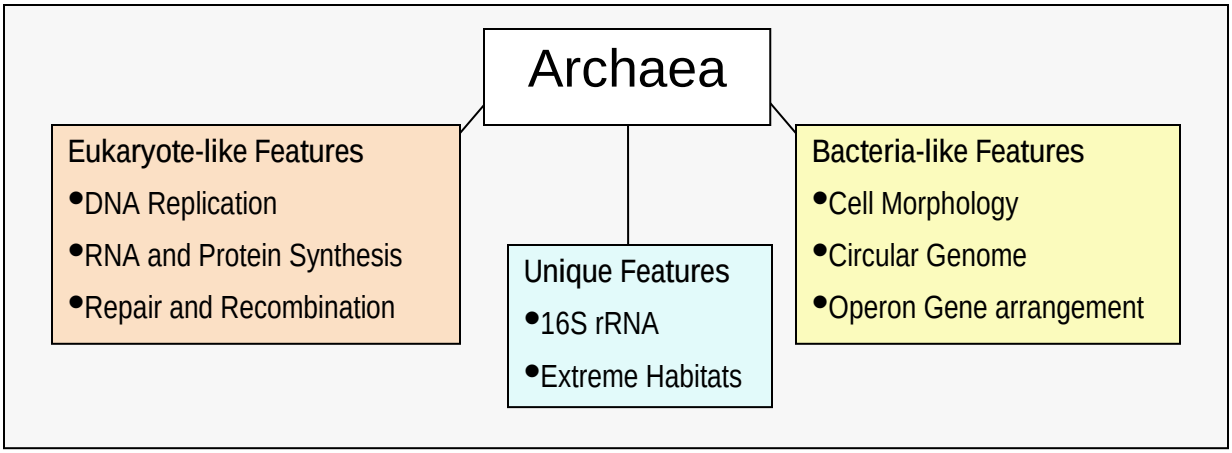

Figure 1.2 Archaea are a combination of bacterial, eukaryotic, and unique characteristics. At first glance they resemble eubacteria due to phenotypic similarities, however further study reveals numerous eukaryotic-like features. Despite the similarities, archaeal 16S rRNA sequences reveal that they are a distinct group of organisms.

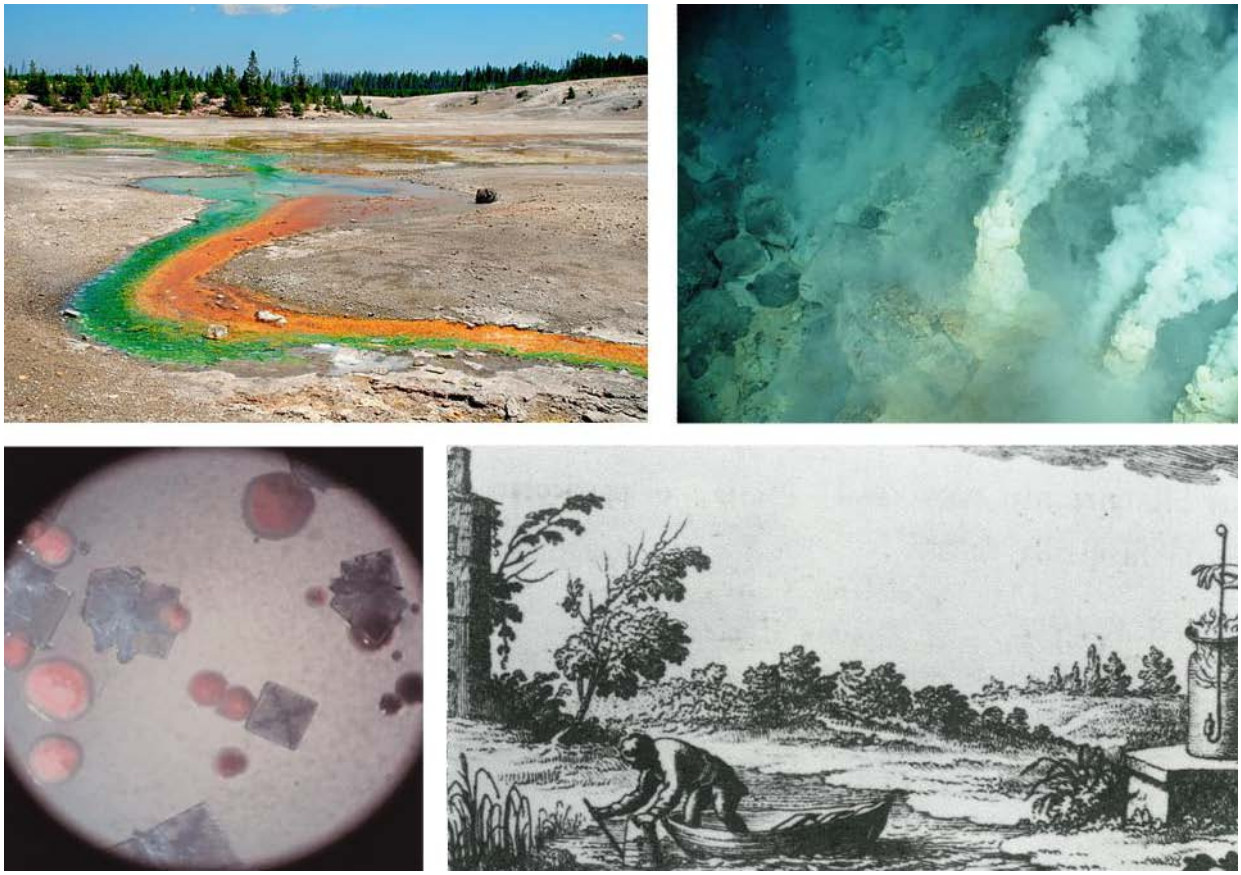

Figure 1.3 The domain *Archaea* encompasses a group of diverse organisms. Top left and right: Thermophilic archaea can be found in hotsprings and are often brightly colored; they also can live at at high pressures and temperatures deep under the ocean near thermal vents (images by L. Rossi and NOAA Submarine Ring of Fire 2004 Exploration and the NOAA Vents Program). Bottom left: Halophiles live at unusually high salt concentrations. Salt crystals sometimes appear on the plates of the halophilic archaeon *Halobacterium NRC-1* (image by P. A. DasSarma). Bottom right: Depiction of early experiments investigating the production of methane in lake sediment. Methane is produced by methanogens, which live in a variety of anaerobic environments, including the lake bottoms and the rumen of cattle (image source[174]).

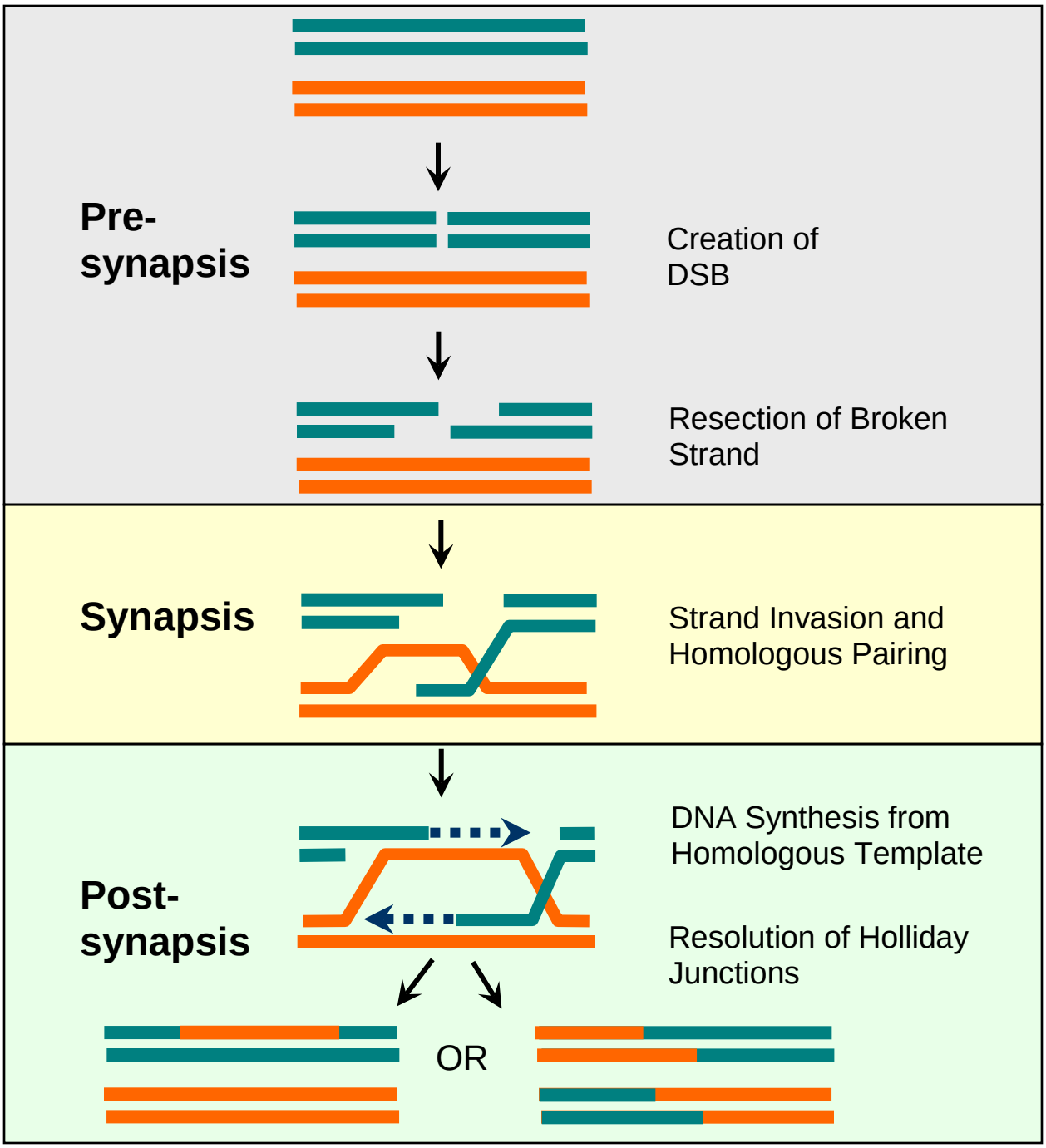

Figure 1.4 Repair of a double-strand break via homologous recombination. Upon creation of a break, the exposed DNA ends are resected, creating exposed 3' ssDNA. A strand exchange protein then loads onto the exposed DNA and forms the presnyaptic filament. It then performs strand invasion, homology search and, upon detection of a homologue, catalyzes strand exchange. Branch migration occurs to extend the

regions of homology, followed by synthesis of missing information to restore lost nucleotides. Finally, the joint molecules are cleaved and the DNA strands are ligated, yielding identical recombinant molecules.

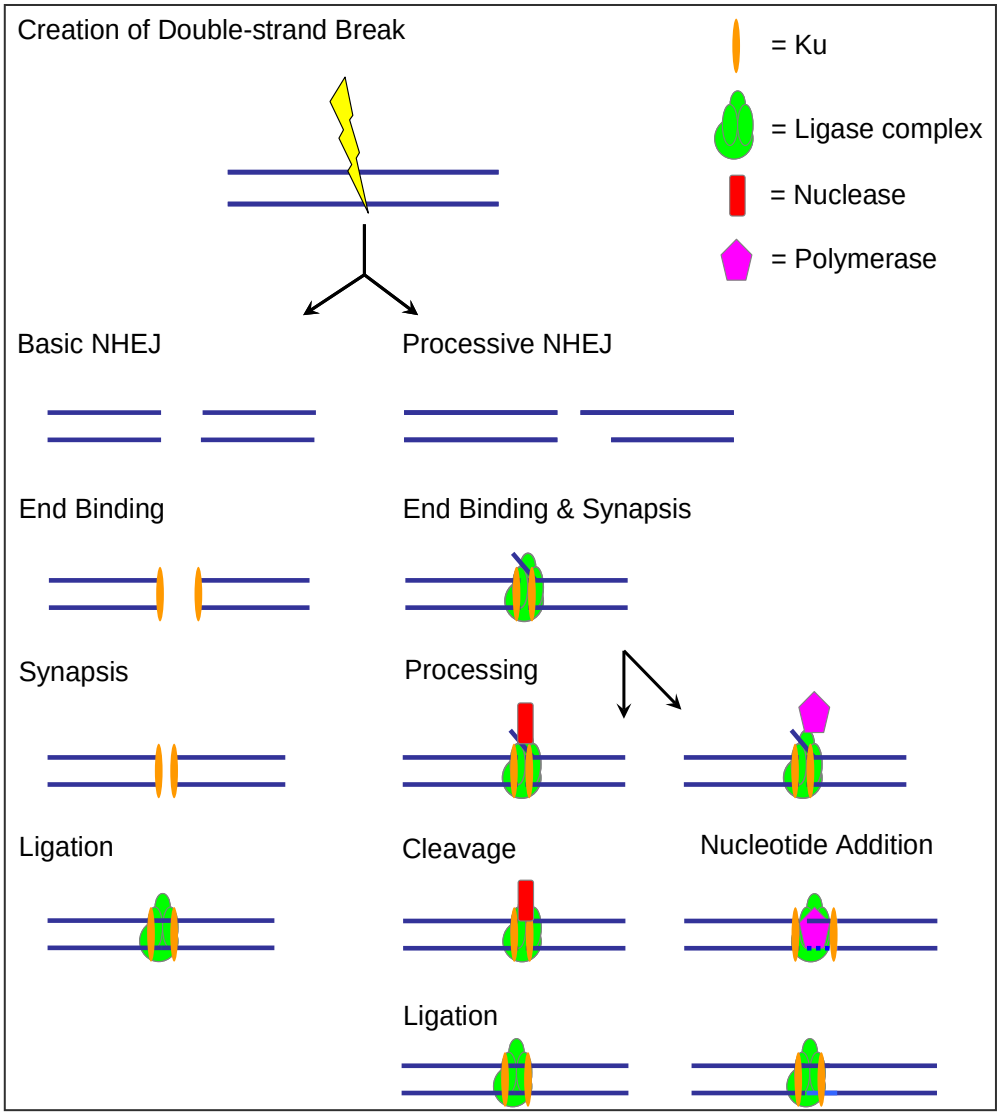

Figure 1.5 Overview of the NHEJ pathway. Basic NHEJ is a simple pathway that involves the bringing together (synapsis) and ligation of compatible broken ends (left). Processive NHEJ will occur if the broken ends are incompatible (right). Nucleases may be used to cleave off overhangs before ligating the ends; alternatively or concurrently, polymerases may fill in extra base pairs to restore missing information or add additional nucleotides. Finally, the mended DNA strands are ligated together.

Table 1.1 Key factors involved in homologous recombination in the three domains of life.

			Archaea	
	Eubacteria	*S. cerevisiae*	Crenarchaea	Euryarchaea
Initiation	RecF/O/R, RecQ, RecJ -OR- RecB/C/D	Mre11/Rad50/Xrs2, Exo1?	Mre11/Rad50? NurA/HerA?	Rad50/Mre11? NurA/HerA?
ssDNA Binding	SSB	RPA	SSB	RPA
Strand Exchange	RecA	Rad51	RadA	RadA
Accessory Factors	None identified	Rad52, Rad59, Rad55/Rad57, Rad54	aRadCs? Rad54?	RadB?
Holliday Junction Resolution	RuvA/B, RuvC	Yen1? Mus81/Mms4? Sgs1/Top3?	Hjc? Hje?	Hjc?

Table 1.2 Key factors involved in characterized NHEJ pathways and hypothetical end-joining factors from the domain *Archaea*.

Function	Bacteria	*S. cerevisiae*	Archaea
End-binding & Synapsis	Ku core	yKu70 yKu80 Mre11/Rad50/Xrs2	Ku core? Mre11/Rad50?
Ligation	ATP-dependent DNA ligase (ADDL)	Dnl 4 Lif1 Nej1	ADDL
Processing: *Nuclease Activity*	Nuclease domain on ADDL	Rad27	?
Processing: *Polymerase Activity*	Polymerase domain on ADDL	Pol 4	?

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CHAPTER 2

ARCHAEAL RAD54 IS INVOLVED IN THE CELLULAR RESPONSE TO UV-INDUCED DAMAGE IN *SULFOLOBUS SOLFATARICUS*

2.1 Introduction

DNA damage repair is a critical capacity for the long term survival of a cell. DNA double strand breaks (DSBs) in particular pose a serious threat to the cell and can lead to cell death if left unrepaired[1]. Homologous recombination (HR) is an error free DSB repair pathway found in all three domains of life; HR is also responsible for the repair of other lesions including single-strand breaks, interstrand crosslinks, and single-strand gaps[2,3,4]. Because double strand breaks are not a common occurrence in most cells, one common use of HR may be to mend damage created by stalled replication fork machinery, which has been hypothesized to occur in 18% of cells undergoing a round of cell division[5,6].

Studies of damage repair pathways initially involve exposure to a selected form of damage in order to investigate an organism's subsequent response. Radiation is one common damaging agent, and the DNA damage produced depends on the energy of the specific kind of radiation used. Ionizing radiation (IR), such as gamma radiation emitted from a ^{60}Co, source is a form of high energy radiation and creates single-strand breaks (SSBs) and DSBs in and exposed organism's DNA. Exposure to IR has long been used as a method of studying HR. Indeed, the factors involved in this pathway were first identified by assessing mutant sensitivity to ionizing radiation[7,8].

Ultraviolet (UV) radiation is a less energetic and more biologically relevant form of damage that leads to a variety of DNA lesions. The most common lesions directly caused by UV radiation are thymine cyclobutane dimers, which can be repaired by the nucleotide excision repair (NER) pathway[9-11]. If NER does not occur by the time replication machinery reaches the lesion, replication stalls, but can resume upstream of the lesion, leaving behind a region of gapped, single-stranded DNA, which can be repaired via

the recombination mechanism[12-16]. In contrast to the damage produced by IR, DSBs are not thought to be directly caused by UV radiation, however studies in archaea and mammals demonstrate accumulation of these lesions following UV exposure[17-19]. These are thought to be byproducts of DNA replication machinery encountering UV-induced ssDNA nicks, and could additionally be substrates for HR[18,19]. Therefore, investigating the involvement of HR following UV irradiation will contribute to current understanding this pathway because it is likely to fix a subset of the resulting lesions.

The mechanism of HR can be summarized as a three step process: presynapsis, synapsis, and postsynapsis[3,4]. Presynapsis involves resection of broken DNA ends and the formation of a nucleoprotein presynaptic filament. Synapsis is the heart of HR and includes the homology search, strand invasion, and joint molecule formation. In postsynapsis, branch migration of the joint molecules occurs. Here, lost information is resynthesized using the homologous template and then joint molecules are resolved.

Synapsis, the central step, is catalyzed by RecA-like strand exchange proteins including bacterial RecA, eukaryotic Rad51, and archaeal RadA[2,3]. Eukaryotic HR utilizes additional Rad51 paralogues and accessory factors to mediate this process that bacterial HR does not use, or at least have not yet been discovered[20-23]. One accessory factor is Rad54, a Swi2/Snf2-like protein and member of the SF2 family of helicases[24,25]. All Rad54 proteins have seven conserved helicase domains, however they cannot perform strand displacement like canonical helicases. Instead, they utilize their ATPase activity to translocate along and remodel dsDNA[22]. *In vitro* studies have led to models in which Rad54 is involved in each of the three major steps of HR, where it interacts with Rad51 and stabilizes the RadA-ssDNA presynaptic filament, promotes D-loop formation, stimulates strand exchange, and catalyzes branch migration of paired substrates[4,22,26-28].

rad51 and *rad54*, like other members of the *rad52* epistasis group, were identified in *Saccharomyces cerevisiae* due to the strong sensitivity of mutants to ionizing radiation, which produces DSBs that are predominately repaired by HR[8,29]. While reports vary, a small but consistent sensitivity to

UV radiation has been demonstrated in *rad54* mutants relative to wild-type[8,30,31]. *rad51* mutants are also mildly sensitive to UV light[7,8,26]. Consistent with these findings, upregulation of *rad51* and *rad54* transcripts following UV irradiation has been documented in yeast and mammals[19,32-34]. To our knowledge, Rad51 and Rad54 protein production following UV radiation exposure has not been reported in these systems.

Recently separated into their own domain within the phylogenetic tree of life, archaea represent a distinct set of organisms characterized by both unique features and similarities to the other two domains[35,36]. Many archaea, particularly those belonging to the phylum *Crenarchaeota*, are constantly threatened with DNA damage due to natural habitats at high temperatures, often accompanied with low pH and exposure to solar radiation[36]. The proposed rate of spontaneous DNA damage in such organisms has been estimated to be orders of magnitude greater than the rate in mesophiles, which has led to interest in archaeal DNA repair mechanisms[37]. Interestingly, studies of the rates of forward (i.e. loss of function) mutations in *Sulfolobus* have indicated similar genomic stability to mesophiles such as *E. coli*[38]. Such organisms must have both efficient and accurate means of repairing constant DNA damage.

Emerging information on archaeal DSB repair has revealed a pathway homologous to eukaryotic HR. Many archaeal recombination genes have higher sequence similarity to eukaryotic genes than bacterial genes[2,23]. Crystal structures of archaeal repair proteins have also led to helpful insights into eukaryotic proteins[39,40]. The archaeal strand exchange protein, RadA, has been characterized and bears closer functional and sequence homology to Rad51 than RecA[41-44]. Interestingly, multiple RadA paralogues have been identified in both archaeal phyla, indicating that RadA activity in archaea may be mediated by accessory factors, similar to the eukaryotic mechanism. These paralogues include RadB in euryarchaea and aRadC (short for archaeal RadC) proteins in crenarchaea[2,45-47]. Despite their homology to RadA, *in vitro* studies of the paralogues do not reveal a clear role for these proteins in HR, making their involvement in this pathway uncertain[45,46].

Of note, the only known prokaryotic Rad54 has been found in the crenarchaeote *S. solfataricus*[2,23,48]. *In vitro* studies of SsoRad54 protein reveal activities that parallel eukaryotic Rad54 protein, including interaction with RadA, stimulation of RadA-mediated strand exchange, and dsDNA dependent ATPase activity[23]. SsoRad54, like eukaryotic Rad54, also remodels dsDNA but lacks true helicase activity[23].

Reports of RadA regulation in response to UV damage are conflicting. Studies of RadA from multiple euryarchaea indicate modest to strong upregulation of transcripts following UV exposure, with one exception, which maybe be due to failure to inactivate the organism's light repair pathway[45,49-52]. A *radA* deletion mutant of *Haloferax volcanii* also demonstrated sensitivity to UV radiation[53]. In contrast, most studies in crenarchaea of RadA transcript regulation following UV exposure report that it is not induced[18,52,54]. Due to its recent discovery, the regulation of Rad54 transcripts in response to UV remains uncharacterized. Determining the transcriptional regulation of *rad54* post-UV exposure would help elucidate HR involvement in the recovery of archaea from this biologically relevant form of damage. It would reveal similarities or disparities with the pathways of other characterized organisms.

Sulfolobus solfataricus is a thermoacidophilic crenarchaeote that can be cultured optimally at 80°C and pH 3 in a laboratory setting[55]. Completion and characterization of the *S. solfataricus* genome and proteome, in addition to the presence of natural viruses and plasmids that have led to the development of a variety of tools which can be utilized for genetic analysis, make this well characterized organism an excellent model archaeon[56-59]. As previously stated, archaeal HR resembles the eukaryotic pathway, and *S. solfataricus* is no exception[2,60]. Studies of *S. solfataricus* have revealed eukaryote-like features additional to those of many other archaea, including prolonged sister chromatid attachment in the G2 phase of replication, replication origination at multiple locations along its chromosome, and the discovery of a Rad54 homologue (described above)[23,61-63]. Thus this organism stands as an excellent model for elucidating archaeal pathways and clarifying complexities within those of eukaryotes.

In this study, we investigate the response of *S. solfataricus* to both ionizing and UV radiation. Following exposure to ionizing radiation, we find that the recovery of three different *S. solfataricus* strains is strain specific. Real time PCR (RT-PCR) analysis of cells exposed to IR indicates that genes encoding proteins involved in HR are upregulated. We also report the recovery following UV irradiation of three strains: *S. solfataricus* 98/2, *S. solfataricus* P2-1, and *S. solfataricus* P2-2, and find again that the recovery is strain specific. We find that *radA* transcripts are upregulated in response to UV damage in all strains, and that *rad54* is upregulated in the derived P2 strains. The transcriptional response following damage appears tightly up and down regulated in the P2 strains, but more gradual in strain 98/2. Further analysis of the *rad54* locus reveals interruptions of the full length gene sequence in two of the strains. Western hybridization analysis of SsoRad54 expression in these strains suggests that their products are not produced at detectable levels, but the full length protein product is detectable in strain P2-1, which does not carry a *rad54* gene insertion.

2.2 Materials and Methods

S. solfataricus strains. *S. solfataricus* 98/2 was obtained as a kind gift from P. Blum, University of Nebraska Lincoln, Beadle Center for Genetics. P2-1 (DSM 1617) was purchased directly from the American Type Culture Collection; it was originally submitted by W. Zillig who isolated it in Italy. P2-2 was obtained as a generous gift from Yvan Zivanovic, PhD, Universite Paris-Sud, Institut de Genetique et Microbiologie.

Cell cultivation and exposure to ultraviolet and ionizing radiation. For return to growth experiments, *S. solfataricus* strains were grown to an optical density of 0.15-0.4 at 540 nm in medium containing 0.2% (wt/vol) sucrose and 0.2% (wt/vol) tryptone (ST medium) prior to IR and UV exposure.

To determine the recovery of *S. solfataricus* to IR, cell samples were exposed to 250, 500, 750, and 1000 Grays of total radiation from a ^{60}Co source emitting gamma radiation at 660 rad/min. Irradiated cells were then added to pre-warmed ST medium and cultivated at 80° C with shaking. Control cells underwent the same manipulations as treated cells but were not exposed to the ^{60}Co source.

For studies of the effect of UV radiation on *S. solfataricus*, cell samples were irradiated in a dark room using an Ultra Lum UVC-508 Ultraviolet cross-linker set to 100, 200, or 300 J/m^2. Irradiated cells were then added to pre-warmed ST medium and cultivated in the dark at 80° C with shaking. Control cells underwent the same manipulations as treated cells but were not exposed to UV light.

For viable cell counts, cells were initially cultured in GT medium (ST medium with 0.2% w/vol glucose substituted for sucrose) to an optical density of 0.33 – 0.48 at 540 nm. They were then irradiated in a dark room using an Ultra Lum UVC-508 Ultraviolet cross-linker set to 100, 200, or 300 J/m^2. Ten-fold serial dilutions of the cells were made in GT medium, and the cells were spotted onto 0.8% GT gelrite plates, allowed to dry, and cultured for five days at 80°C in a humid chamber. Unexposed control cells underwent the same manipulations as exposed cells but were not UV irradiated.

RNA isolation and cDNA preparation. 10 ml samples were removed from cultures at the times indicated. Cells were pelleted by centrifugation at 10,500 x g. RNA was then isolated using the RiboPure-Bacteria kit (Ambion) according to the manufacturer's protocol. Contaminating DNA was removed from RNA samples using the DNA-Free kit (Ambion) following the manufacturer's protocol. cDNA was prepared from DNA-free RNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas) with random hexamer primers and the manufacturer's recommendations. Resulting nucleic acid samples were quantified at 260 nm using a Coulter Beckman DU-800 Spectrophotometer. Experiments were performed in triplicate from either one or two RNA isolations from each strain.

Polymerase Chain Reaction (PCR). PCR was performed on genomic DNA isolated from each strain *S. solfataricus.* The primers used were 5' CGGGATCCATCATCAATTTCTTCTCTTA-TATTCTTTCC-3' and 5'-CGGGATCCCTTAGCTCTTTGTGAAAATTTAACTAATCC-3'. After an initial 2-5 minute predenaturation step, genomic DNA was melted at 94°C for 30 seconds, followed by primer annealing for 30 seconds at 60° C, and DNA synthesis for 4 minutes at 72°. PCR products were run on 0.8% 1 x TBE agarose gels and stained in dilute ethidium bromide to resolve the product lengths. Contrast adjustment of the resulting images was performed using Microsoft Power Point image editor.

Peptide antibody preparation. Antibodies against a synthetic peptide fragment corresponding to residues 560 – 573 in the full length ssoRad54 amino acid sequence were produced in rabbits by YenZyme Antibodies, LLC.

Western hybridization analysis. *S. solfataricus* samples to be analyzed were grown in ST medium to exponential growth phase and harvested via centrifugation. Cell pellets were resuspended in TNGS (20 mM Tris-Cl, pH 7.5, 1 M NaCl, 10% glycerol, 0.25% N-lauryl sarcosine) buffer and sonicated to disrupt cell

membranes and chromosomal DNA. Total protein concentration was then determined via absorbance readings at 280 nm. Sonicates were directly analyzed or stored at -80°C for future use by first flash freezing in a dry ice/ethanol bath. Equal protein concentrations were loaded and electrophoresed using 4% stacking and 8% separating acrylamide tricine gels; the anode buffer was composed of 200 mM Tris-Cl, pH 8.9 and the cathode buffer was composed of 100 mM Tris, 100 mM tricine, and 0.1% SDS. Samples were then transferred to nitrocellulose membranes for one hour at 350 mA in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol) using a BioRad Mini TransBlot cell and blocked overnight in TBS (10 mM Tris-Cl pH 8.0, 150 mM NaCl) buffer supplemented with 5% dried milk. All incubations of the hybridized membrane were carried out at room temperature. The following day transferred membrane were briefly washed with TBST (TBS plus 0.05% Tween 20) buffer and probed for 3.5 hours with 1:3,000 anti-Rad54 antibody in TBST buffer. Blots were again washed with TBST buffer and then probed for approximately one hour with 1:10,000 horseradish peroxidase conjugated Goat anti-Rabbit IgG antibody (Pierce). Blots were exposed using Pierce ECL Western Blotting substrates following the manufacturer's instructions and exposed to X-ray film. Contrast adjustment of exposed films was performed using Microsoft Power Point image adjustment tools.

2.3 Results

2.3.1 Recovery of S. solfataricus *to ionizing radiation is strain specific.*

Four strains of *S. solfataricus*: 98/2, P2-1, and P2-2, were exposed to gamma radiation to assess whether recovery from a source that directly produces DNA DSBs is strain specific. Cells were cultured in rich medium until they reached exponential phase growth, damaged with 250, 500, 750, and 1000 Grays of ^{60}Co radiation, and subcultured into fresh, prewarmed medium. Recovery was monitored by optical density readings at 540 nm. Figure 2.1 depicts recovery curves that are representative of three replicate experiments performed for each dose for each strain.

Although all four strains returned to exponential growth immediately after the lowest dose of radiation (250 G), and all strains were unable to recover from the highest dose (1000 G) after 100 hours or more (i.e. twenty or more doublings), results from intermediate levels of damage indicated strain specificity. Strains P2-1 and P2-2 recovered similarly to each other. Following 500 G of irradiation, they experienced lagging growth for around ten hours, corresponding to about one to two doublings before resuming exponential growth. 750 G of irradiation resulted in slowed growth until ninety hours following exposure, or approximately twenty doublings. In contrast, stain 98/2 experienced an approximately forty hour lag from a 500 G dose, corresponding to about ten doublings. Interestingly, its recovery to 750 G was similar to the P2 strains: it resumed logarithmic growth in less than 80 hours, or approximately eighteen doublings.

2.3.2 Recovery of S. solfataricus *to ultraviolet radiation is strain specific.*

To determine the effect of UV radiation on *S. solfataricus* growth, and to examine the possibility of strain specific recovery, *S. solfataricus* 98/2, P2-1, and P2-2 were grown to exponential phase in rich media and each exposed to 100, 200, and 300 J/m^2 of UV radiation. Exposed cells were subcultured into fresh, prewarmed media and recovery was monitored as a function of the optical density at 540 nm. Figure 2.2

depicts recovery curves that are representative of three replicate experiments performed for each dose for each strain.

The responses of the three strains had similarities and differences. Each culture recovered quickly from the lowest doses of radiation, 100 J/m^2 and 200 J/m^2. The doubling times of all cells exposed to 100 J/m^2 were lower relative to the control, but exponential growth was maintained. Likewise, after 200 J/m^2 of UV radiation, all strains recovered after about 40 hours, or eight to nine doublings. Strain specificity was apparent in cell exposed to 300 J/m^2. *S. solfataricus* strain P2-1 and P2-2 cells irradiated with 300 J/m^2 resumed logarithmic growth shortly after cells irradiated with 200 J/m^2, by about 50 hours or just over ten doublings. In contrast, *S. solfataricus* strain 98/2 was not able to recover from 300 J/m^2 within 100 hours of incubation time following UV irradiation. The similarities in P2-1 and P2-2 recovery were expected due to their origination from the same isolate, unlike 98/2 which was isolated from an entirely different location.

Viable plate counts supported the evidence that the recovery of all strains was similar following 100 J/m^2 and 200 J/m^2 of UV radiation. Indeed, there was no significant difference in the percentage of surviving cells at these doses. While large standard deviations suggest insignificant difference in survival from 300 J/m^2, the average percent survival reflects the data obtained in the return to growth studies. Aggregation in response to UV damage has previously been documented, as have large standard deviations for viable plate counts in both exposed and unexposed cells[18].

The lowest dose, 100 J/m^2, was chosen for future experiments because damaged cells were able to easily resume growth following irradiation, indicated by their immediate return to growth. Average viable plate counts revealed 11 – 24 % survival of *S. solfataricus* cells to this level of UV exposure.

2.3.3 radA *and* rad54 *transcripts are upregulated in response to IR and UV damage.*

To further investigate strain specific differences between strains 98/2 and P2, we investigated the abundance of transcripts of genes involved in HR following ionizing and UV radiation damage. Quantitative

RT-PCR results demonstrated that changes in the levels of *S. solfataricus radA* and *rad54* transcripts were strain specific (Figure 2.4). First, it was noted that *radA* transcripts increased in abundance in all strains following both kinds of damage, peaking at approximately 30-45 minutes post exposure. A greater change in transcript levels was noted in the P2 strains and varied dramatically with the form of damage. *radA* transcripts reached nearly forty fold over their constitutive levels following IR and approximately six fold greater levels following UV radiation. IR is a known source of double-strand breaks, while UV typically produces thymine dimers and double-strand breaks are believed to appear only from inability to properly repair them before replication occurs. Therefore, these results are consistent with a major role for HR in the repair of IR-induced damage and a lesser role in the repair of UV-induced damage, according to the levels of DSBs that are believed to be produced by each. Strain 98/2 *radA* transcripts were also higher in abundance following IR than they were following UV, reaching approximately five and three fold over the untreated control, respectively. The higher transcript abundance observed in *radA* transcripts following exposure to UV and ionizing radiation supports the involvement of HR in repair of their resulting DNA lesions.

rad54 transcript levels increased in a strain and damage specific manner. Again, *rad54* transcripts were more abundant in treated cells than they were in untreated cells following both UV and ionizing radiation, however the increase in abundance was the greatest following IR, which induces direct double-strand breaks. Interestingly, the results were very different for strain 98/2: *rad54* transcripts were eight fold more abundant following ionizing radiation, however they changed very little following UV radiation. The varying levels of *rad54* transcripts in particular led to the hypothesis that there is a physiological explanation for the strain dependent regulation of *rad54* in response to UV.

2.3.4 Different strains of S. solfataricus *maintain* rad54 *genes of different lengths*

rad54 was amplified from genomic DNA prepared from *S. solfataricus* 98/2, P2-1, and P2-2, revealed differently sized products (Figure 2.4A). The gene amplified from P2-1 corresponds to a full length uninterrupted gene, which is 2721 bp (Figure 2.4B)[23]. Translated, this would result in a 104.6 kDa protein containing 906 residues. The major product amplified out of P2-2 is approximately 1 kb larger in size. This size increase is due to the presence of a 966 bp transposable insertion (IS) element located approximately 2.4 kb from the 5' end of the gene. The sequence has been annotated as three separate open reading frames (SSO1653-SSO1655) in the published genome sequence for this strain[58]. The IS element results in the addition of forty-two premature stop codons in the coding sequence, suggesting the resultant protein would contain 802 residues and have a molecular weight of 92.8 kDa. *rad54* amplified from strain 98/2 was also larger than the uninterrupted gene. Sequence analysis revealed a 348 bp insert present approximately 1.8 kb from the 5' end of the gene. This interruption leads to 40 premature stop codons in the coding sequence, suggesting that translation of the transcript would yield a truncated product with 605 residues and have a molecular weight of 70.7 kDa.

2.3.5 SsoRad54 protein is only detected from strain P2-1

To determine if 98/2, P2-1, and P2-2 produce Rad54 protein products of variable sizes corresponding to the early stop codons in their gene sequences, western hybridizations were performed on whole cell extracts prepared from each strain. The anti-Rad54 peptide antibody used for the primary antibody in the hybridization was raised against a sequence fragment sufficiently early in the protein that it would allow detection of both truncated and full length protein products. Figure 2.5 depicts representative results from at least three replicate protein preparations from each strain. Full length Rad54 (lane 2) was repeatedly detected in P2-1 whole cell extracts and ran slightly faster than heterologously purified Rad54 (lane 1) which may either be due to the absence of a His tag and/or failure to completely denature the

native protein. Rad54 protein products were not detected from *S. solfataricus* strains P2-2 or 98/2, indicating that they are either produced levels below the detection limit, produced but quickly degraded, or not produced at all.

2.4 Discussion

To our knowledge, this is the first report of the recovery of *S. solfataricus* after IR exposure and the first comparison of multiple strains' responses to UV irradiation. Ionizing radiation is a known source of DSBs that can be mended either by the HR or nonhomologous end-joining pathways[29,64]. Although less characterized, HR has also been found in archaea, and emerging information suggests the pathway resembles that of eukaryotes more than eubacteria[60]. As expected, the recovery of all four *S. solfataricus* strains to IR was dose dependent. The time it for strains P2-1, P2-2, and 98/2 to return to exponential growth was similar, although an extended lag in the recovery of strain 98/2 to 500 G suggests this strain may have slightly impaired recovery relative to the P2 strains.

UV radiation predominately creates thymine dimers that can ultimately be processed into DSBs, SSBs, and single-strand gaps[9,13,16]. While UV lesions can be mended by the NER pathway, the detection of accumulated DSBs following UV damage suggests that HR may play a role in recovery from UV as well[11,18,19,65]. Homologues of proteins involved in eukaryotic NER have been identified in all archaea and homologues of bacterial NER factors have been found in euryarchaea only[66,67]. While the bacterial NER homologues appear to have a role in dark repair of UV damage in euryarchaea, the presence of a functional NER pathway in crenarchaea has not been established[66]. A recent study looking for transcription coupled repair in *S. solfataricus* found no evidence of accelerated thymine dimer repair in transcribed DNA[68]. Given the uncertainty of an NER pathway in *Sulfolobus*, the investigation of HR's role in fixing UV-induced lesions becomes even more important.

UV damage is biologically relevant and easy to perform in a laboratory setting; consequently, the UV damage response has been documented in many model organisms, including *Sulfolobus*, a model crenarchaeal genus. The majority of studies report that DNA repair genes in this organism – including RadA, the central strand exchange protein of HR – are not induced following UV irradiation[18,54,67]. These findings are surprising due to contrary reports in euryarchaea that find modest to strong upregulation of

Rad A transcripts following UV exposure[49-52]. Furthermore, progressive elucidation of archaeal HR has revealed many similarities to the eukaryotic pathway, and upregulation of Rad51 transcripts and promoters in response to UV damage has been documented in eukaryotes[32,33]. The discovery of a Rad54 homologue in *S. solfataricus* has introduced a new means of detecting HR in this archaeon. Here, we report the first comparison of the recovery of thee strains of *S. solfataricus* to UV radiation. We also report the upregulation of *rad54* and *radA* transcripts in response to UV and ionizing irradiation. Further investigation of the *rad54* locus in each of these strains reveals a different sized *rad54* gene in each strain. Western hybridization analysis reveals that a Rad54 protein product is only detectable in strain P2-1, which is the only strain bearing the full length, undisrupted gene.

Analysis of strains P2-1, P2-2, and 98/2 suggests a degree of strain specificity in the response of *S. solfataricus* to UV radiation. Specifically, strains P2-1 and P2-2 are able to recover from UV damage more quickly than strain 98/2 at a high dose of radiation. Strain dependent responses to DNA damage has not yet been reported in *Sulfolobus.* The genomes of both strains have either been published or submitted for publication and a striking difference between the two is the high population of IS elements (approximately 11% of the genome) in P2 relative to 98/2 (approximately 5% of the genome)[58,69]. The physiological repercussions of maintaining such a large number of IS elements have not been established, however the potential threats are obvious: random jumping of mobile elements into the middle of essential genes could cause cell death. We hypothesize that P2 has adapted to the threat of IS hopping by maintaining tight control of its genome. RT-PCR results support this hypothesis. Our results demonstrate that transcription of *radA* and *rad54* in P2 can be turned on and off in 15 minutes, whereas transcription is less tightly controlled in strain 98/2, evidenced by increased levels of *radA* transcripts from 30 to 60 minutes following UV exposure.

This is the first detailed report of increased transcript abundance of *radA* and *rad54* following IR and UV damage. Specifically, it is the first investigation of *S. solfataricus* transcript levels of genes

involved in HR following IR, and the higher abundance of *radA* and *rad54* transcripts supports a hypothesis of for the involvement of their protein products in DSB repair. Secondly, this is the first supported evidence for the involvement of HR in the repair of lesions produced either directly or indirectly from UV damage in our microorganism. Our finding that *radA* is upregulated in response to UV damage differs from previous reports, however it does not necessarily conflict with them. RT-PCR results from both *S. solfataricus* strains P2 and 98/2 confirm that upregulation of important HR genes occurs immediately after UV exposure; failure to detect RadA upregulation in previous studies may be due to waiting too long to probe for abundance. Additionally, studies of mRNA half lives in *S. solfataricus* indicate that transcripts can persist in the cell up to two hours however the median half life is estimated to be approximately five minutes[70,71]. Results from microarray analysis specifically estimate the half life of Rad54 to be under eight minutes[70]. Our data, in conjunction with these previous findings, indicates a distinct possibility of missing detection of transcripts or protein products by using solely 30 minute time points. Another possible explanation for differential results is dosage. Several studies use a higher dose (200 J/m^2) of UV to damage their cells[54,67]. Viable plate counts performed in our lab indicate that 98-99% of *S. solfataricus* cells are often killed by this exposure. Additionally, transcriptional regulation may be different at such a high level of damage, as has been suggested by previous findings in euryarchaea[49-51]. Thus, differential reports may be largely due to procedural differences.

Further examination of the *rad54* locus revealed inserted sequences within the *rad54* genes of strains P2-2 and 98/2. Such findings have not been reported elsewhere, however point mutations leading to missense mutations in the amino acid sequence have been discovered in *rad54* genes of several cancer patients[72]. Creation of a corresponding mutation in yeast gave the mutant sensitivity to DNA damaging agents such as methyl methane sulfonate (MMS) and UV radiation[73]. *In vitro* analysis of the mutant protein revealed impaired ATPase activity and ability to translocate along DNA, although it was still able to bind DNA and interact with Rad51[73]. The insertions found in the *rad54* gene of strains P2-2 and 98/2 would lead

to truncated products that maintain Walker A boxes responsible for ATPase function, but would be missing two or three of the seven conserved helicase domains of SWI2/SNF2 proteins, respectively[48]. *In vitro* studies indicate that the N-terminal portion of eukaryotic Rad54 is responsible for Rad51 interactions, thus the RadA-SsoRad54 interaction may not be disrupted with the truncated proteins[74,75]. Mutational analyses have been performed on the seven helicase domains of yeast Swi2/Snf2, a member the of Swi2/Snf2-like protein family to which Rad54 belongs[76]. Reporter assays of mutants indicate that there are critical residues within each helicase domain that are responsible for protein function[76]. While truncation studies have not been performed, these findings indicate that the entire loss of two or three of these domains could severely impair SsoRad54 function.

Western hybridization analysis was able to detect full length SsoRad54 in strain P2-1 but was unable to detect SsoRad54 protein products of any length in either strains P2-2 or 98/2. Three explanations could justify these findings: first, inserted *rad54* transcripts are degraded before they are translated into proteins and truncated SsoRad54 products are never produced, second, truncated SsoRad54 is produced but immediately degraded by the cell, or third, truncated proteins are produced at sub-detection levels. Enzymes by definition are recycled proteins, thus the cell would require fewer of them present than proteins with non-enzymatic functions, such as filament forming SSB. Indeed, we find that SsoRad54 is present in very low abundance in *S. solfataricus*, and the inability to detect an even lower level of truncated product with our current system is a distinct possibility.

Natural mutations leading to loss of Rad54 protein in an organism have not previously been reported. While it is well known that *rad54* is not an essential gene, *in vitro* studies performed in eukaryotes have indicated that this protein is likely involved in HR from presynapsis to postsynapsis[77]. Eukaryotic organisms utilize Rad54 homologues, such as Rdh54 in *S. cerevisiae*, that are believed to serve primarily in meiotic recombination and could potentially assist in mitotic recombination in the case of Rad54 functional loss[78]. No SsoRad54 homologues have been found in *S. solfataricus*, however. How an

organism isolated from an environment naturally conducive to continual DSBs from solar radiation and high temperatures could naturally lose and maintain loss of a protein with so many functions within a critical damage repair pathway will be a topic for future investigation. RT-PCR results indicate significant increases in transcript abundance of *rad54* following UV damage in the P2 strains and very change in *rad54* transcripts in strain 98/2. These results make sense for strains P2-1 and 98/2. As already discussed, *rad54* transcripts levels of full length, undisrupted genes have been previously found in eukaryotes to increase following UV damage[19,30,34]. Likewise, it is logical that strain 98/2 *rad54* transcripts would not increase if the transcript or protein product is degraded – to do so would be unproductive for the cell. Interestingly, *rad54* transcripts did increase in abundance following ionizing radiation; this may indicate that degradation occurs at the protein level rather than at the RNA level. Collectively, our results suggest that the difference in survival to radiation is not due to *rad54* status.

Preliminary western hybridization results of SsoRad54 protein levels in strain P2-1 from fifteen to sixty minutes post-UV exposure are equivocal. While some results suggest an upregulation in protein expression around thirty minutes, other results indicate that SsoRad54 levels are instead dropping. More replicates of these experiments will be required to elucidate what is truly happening to SsoRad54 levels within the cell following UV exposure. It is hypothesized that protein levels will increase in a manner that corresponds with the increase observed in *rad54* transcripts, however this may be difficult to detect due to fact that approximately 90% of the cells are killed at this dose of radiation.

Taken together, these results present new insights into the response of *S. solfataricus* to ionizing and ultraviolet radiation. Strain comparisons of the recovery of this organism to DNA damage reveal strain specific differences which are likely to have genetic bases. RT-PCR results further suggest strain specific responses, but are consistent in demonstrating for the first time the increased abundance of transcripts of genes involved in HR following DNA damage. We also show for the first time evidence of the involvement of SsoRad54 in repair to UV and ionizing radiation induced lesions. We find that this protein is likely a

nonessential protein in *S. solfataricus* due to its apparent absence in two of the observed strains, however the full length protein is produced and *rad54* transcripts are upregulated in response to UV radiation. Eukaryotic Rad54 protein has been previously studied in eukaryotes and appears to have multiple roles in HR. Future characterization of this archaeal homologue will elucidate its roles in the archaeal HR pathway.

2.5 Acknowledgements

Dr. Michael Rolfsmeier provided the viable plate count and RT-PCR data shown in this work and will be a co-author on the manuscript that will be submitted for publication.

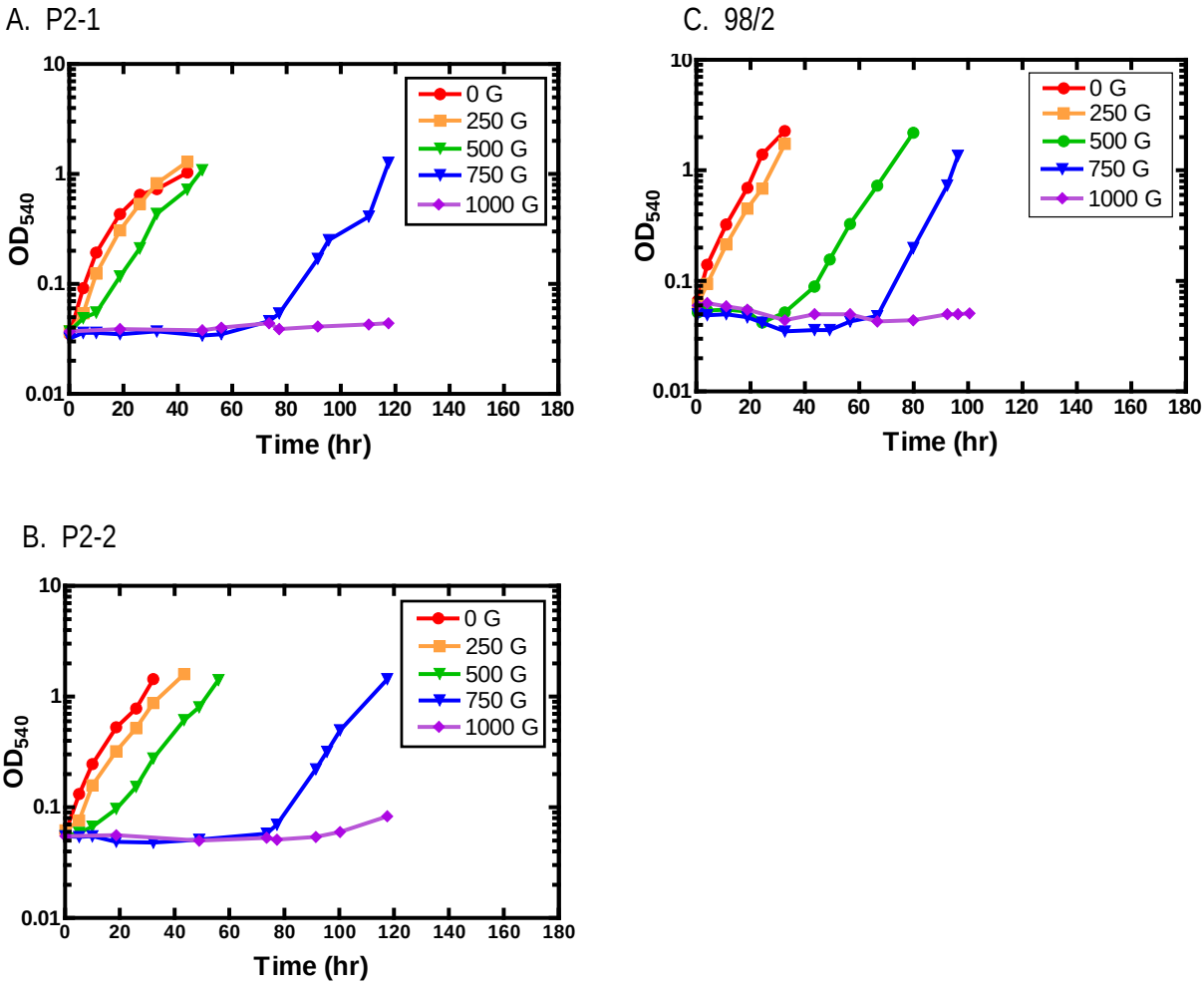
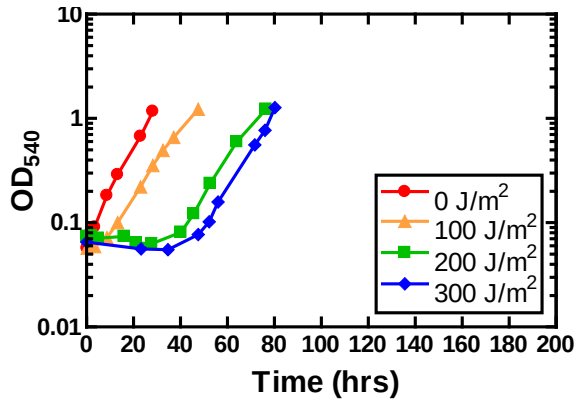
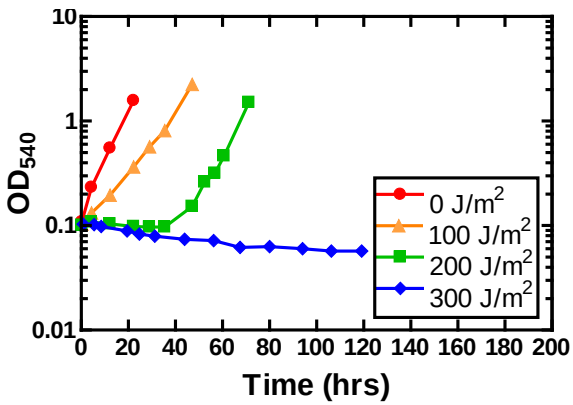


Figure 2.1 Return to growth of *S. solfataricus* after exposure to gamma radiation is strain specific. Representative growth curves of UV-irradiated *S. solfataricus* strains (A) P2-1, (B) P2-2, and (C) 98/2 are shown above. Exponentially growing cultures of *S. solfataricus* were exposed, subcultured into rich medium at zero hours, and monitored for growth at a wavelength of 540 nm at the indicated time points.

A. P2-1



C. 98/2



B. P2-2

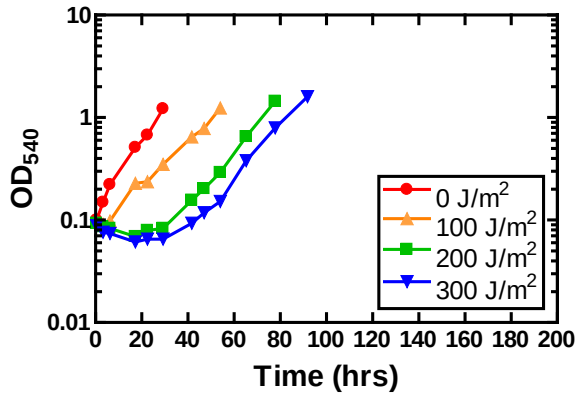
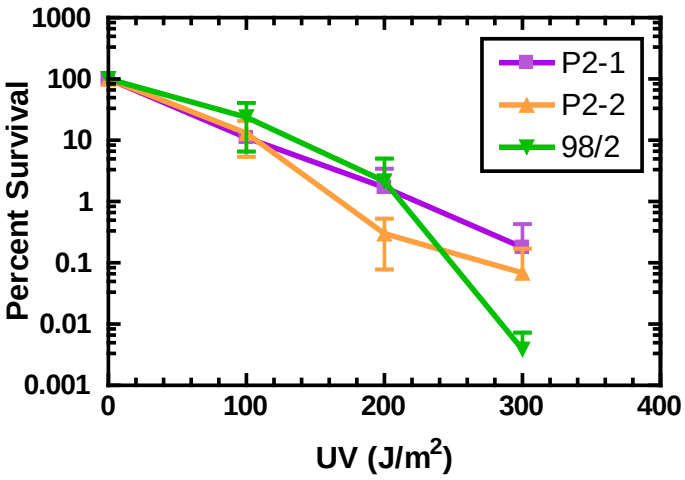


Figure 2.2 Return to growth of *S. solfataricus* after exposure to UV radiation is strain specific. Representative growth curves of UV-irradiated *S. solfataricus* strains (A) P2-1, (B) P2-2, and (C) 98/2 are shown above. Exponentially growing cultures of *S. solfataricus* were exposed, subcultured into rich medium at zero hours, and monitored for growth at a wavelength of 540 nm at the indicated time points.

A.



B.

	P2-1	P2-2	98/2
100 J/m^2	11.0 ± 2.4	13.1 ± 7.7	23.8 ± 17.2
200 J/m^2	1.4 ± 1.7	0.30 ± 0.22	2.2 ± 2.9
300 J/m^2	0.20 ± 0.25	0.069 ± 0.1	0.0039 ± 0.0034

Figure 2.3 Viable plate counts of *S. solfataricus* strains P2 and 98/2 exposed to UV radiation. Cell counts reveal insignificant differences in the survival of all three strains to doses of 100 and 200 J/m^2. Survival data for a 300 J/m^2 exposure also reflects return to growth data.

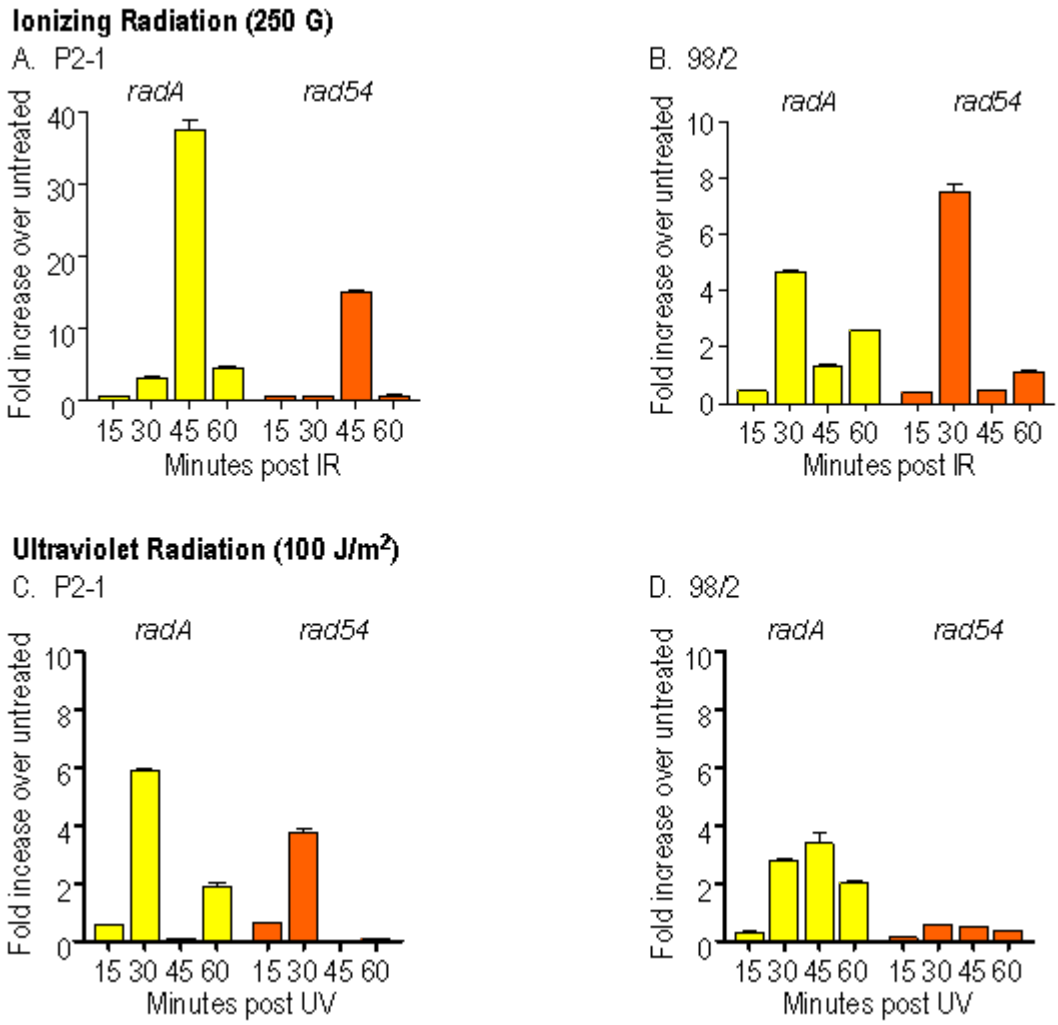


Figure 2.4 *S. solfataricus radA* and *rad54* transcripts are upregulated in response to UV and ionizing radiation. Quantitative RT-PCR was used to assess transcript levels following DNA damage exposure. Transcript levels were determined based on the abundance of the 23S ribosomal RNA housekeeping gene transcripts. Higher abundance of both *radA* and *rad54* transcripts was detected in strain P2-1 (A) and strain 98/2 (B) following IR, peaking at 45 and 30 minutes, respectively. Significant increases in both *radA* and *rad54* transcripts were seen following UV exposure in strain P2-1 at 30 minutes post exposure (C). *rad54* transcripts did not change in abundance following UV irradiation in strain 98/2, however *radA* transcript levels increased from 30 to 60 minutes post exposure (D). P2-2 results were similar to those found for P2-1 (data not shown).

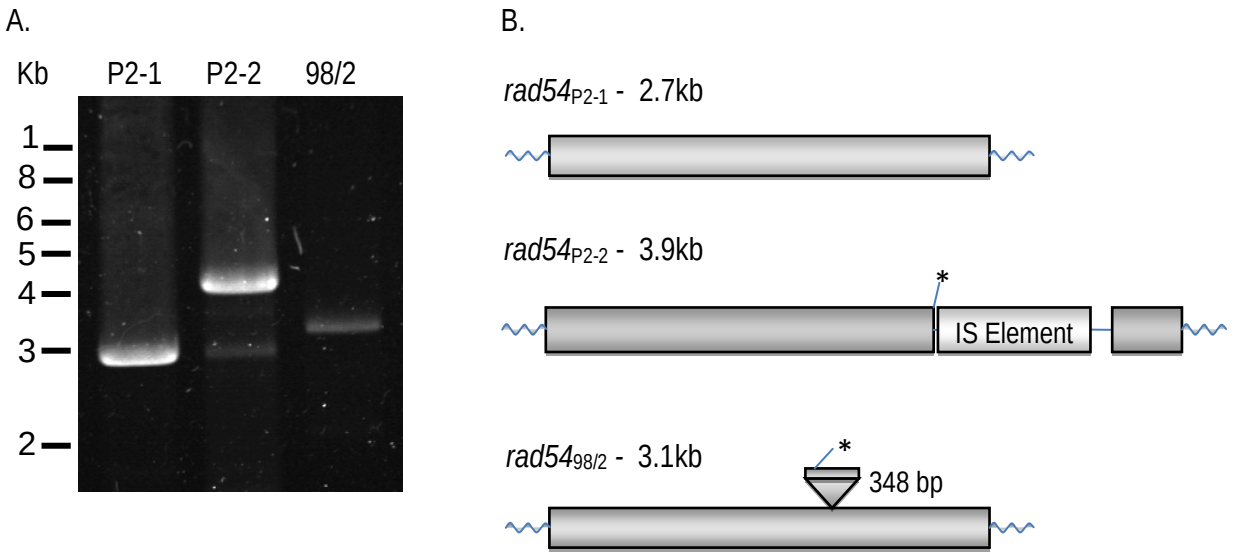


Figure 2.5 *rad54* is disrupted in *S. solfataricus* strains P2-2 and 98/2. (A) *S. solfataricus rad54* specific primers were used to PCR amplify the gene from genomic DNA. (B) The amplified products indicate size differences in strain P2-2 due to a 966 bp IS element and in 98/2 due to a 348 bp inserted sequence. In both cases, sequence analysis reveals a series of premature stop codons encoded within the gene due to the inserted sequence. The first of these is marked with an asterisk (*).

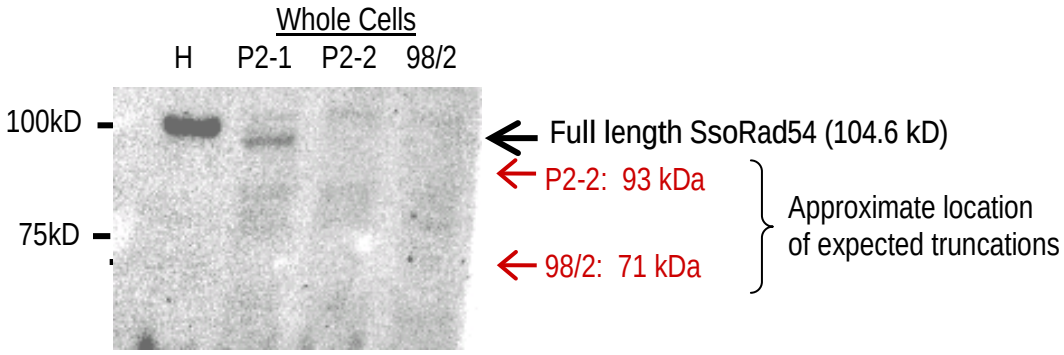


Figure 2.6 Detection of Rad54 protein *in vivo.* Whole cell extracts of *S. solfataricus* strains P2-1, P2-2, and 98/2 were prepared from exponentially growing cultures and probed with a peptide antibody raised against residues 560-573 of the SsoRad54 amino acid sequence, which allowed for detection of truncated and full length products. Strains are indicated above their corresponding lane, H indicates heterologously expressed protein purified as described[23].

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CHAPTER 3

DEVELOPMENT OF *IN VITRO* AND *IN VIVO* ASSAYS TO TEST FOR END-JOINING IN THE ARCHAEON *SULFOLOBUS SOLFATARICUS*

3.1 Introduction

Double-strand breaks (DSBs) are one of the most dangerous lesions that threaten DNA. Not only do they destabilize the genome, but they can also trigger apoptosis; a single DSB can cause cell death if left unrepaired[1]. Such lesions are dangerous because they are inherently difficult to repair due to the potential separation of the broken ends. If other double-stranded DNA ends are simultaneously available, rejoining with an inappropriate partner may occur, leading to chromosomal aberrations like translocations and rearrangements[2]. DSBs can also be deliberately created via endogenous pathways such as V(D)J recombination and meiotic homologous recombination for the specific purpose of promoting genetic diversity. Given the multiple sources of this lesion, an estimated 10 - 100 endogenous DSBs per nucleus per day occur in a mammalian cell[3].

To combat such constant attacks, cells are equipped with pathways that specifically repair this form of DNA lesion. Two of these pathways are the homologous recombination (HR) and nonhomologous end-joining (NHEJ) pathways. Whereas HR utilizes a homologous DNA template to perform error free repair, NHEJ does not use a template and is more likely to be error prone. Due to template requirements, HR is believed to be the predominant DSB repair pathway in cells with a newly replicated chromosome or sister chromatid in close proximity (e.g. S or G2 phase) while NHEJ repair predominates when a template is not available (e.g. G1 or G0 phase)[4]. NHEJ is the primary DSB repair pathway in mammals and is estimated to be 10 – 100 times more efficient than HR in mitotic mammalian cells[5]. NHEJ is not only critical in the repair of unwanted DNA damage but it is the key method of repair of deliberate DSBs. These are commonly generated by recombination activating nucleases which cleave DNA at specific sites in order to

stimulate NHEJ-mediated rejoining via V(D)J recombination[6]. Rearrangements mediated through V(D)J recombination result in a diverse library of antigen receptors that equip lymphocytes in the human immune system[7].

The NHEJ mechanism involves binding broken DNA ends, bringing them together (known as *synapsis*), processing (if ends are incompatible), and religation[8]. The pathway was initially discovered and remains best characterized in eukaryotes, however it has now also been found and studied in eubacteria[9,10]. Two NHEJ factors are conserved from bacteria to mammals: a Ku dimer and an ATP-dependent DNA ligase. While ligases are used ubiquitously throughout biology, Ku proteins are not. Thus, the presence of Ku proteins has become the key identifier of NHEJ in an organism and indeed led to the discovery of NHEJ in bacteria[11,12].

Ku is a DNA binding protein that has a preference for binding DNA ends[13,14]. The crystal structure of eukaryotic Ku explains this preference by demonstrating that it forms a ring-shaped complex that threads itself onto DNA[13,15]. Ku binds broken ends immediately following the creation of a DSB and may play a role in synaptic step of NHEJ as well[16-18]. Ku also interacts with other factors involved in NHEJ, such as the DNA ligase, although this interaction may be DNA mediated[19,20]. Kinetic studies in budding yeast have demonstrated that ligase is recruited to the DSB after Ku, where simple religation of the break can occur if the DNA ends are compatible[16]. If broken ends are not compatible, processing is necessary for NHEJ to be completed. Both eukaryotes and prokaryotes utilize polymerases and nucleases to fill in gapped DNA or cleave overhanging flaps, respectively. In bacteria, this function comes from additional activities of the ATP-dependent ligase whereas eukaryotes use additional protein factors to do so[1,10].

The regulation of Ku and ligase transcripts following UV or ionizing radiation is not well characterized in yeast or mammals[21]. It also has not been reported in eubacteria to our knowledge. Most of the studies in eukaryotes that have emerged are based on microarray analyses and suggest that Ku and ligase transcripts are not upregulated in response to UV and IR, however reports are conflicting, as Ku

upregulation has been reported in response to IR by Otomo, *et al*. in glioblastoma cells[22-24]. Further studies are necessary to clarify the regulation of NHEJ factors in response to DSBs.

An impaired NHEJ pathway has catastrophic repercussions. Mutations have been found and characterized in several NHEJ factors in humans and all result in severe combined immunodeficiency (SCID) conditions[25-29]. Hallmark characteristics of the resultant disease include immunodeficiency, growth retardation, and growth malformation. Additionally, DNA analysis of cell lines often reveals chromosomal aberrations. NHEJ deficient humans often die prematurely from either cancer (e.g. lymphomas) or recurrent infections.

A thorough understanding of this pathway is critical step towards treating disease, yet current knowledge of NHEJ is lacking. Current understanding of the *in vivo* functions of key proteins remains deficient. For example, Ku appears to be involved in include protection of ends, synapsis, and factor recruitment, however these roles are unsettled[18,30-33]. The mechanism of end ligation is also disputed due to evidence that DNA strands may be ligated sequentially rather than simultaneously[32,33]. Eukaryotic systems are additionally complicated by the multiple roles that their factors have within the organism. Many of the proteins involved in NHEJ have in other cellular functions besides this repair pathway. Ku, for example, appears to additionally function in telomeric length maintenance and transcriptional regulation[34-36]. Multi-use enzymes complicate *in vitro* findings because it can be difficult to correctly correlate *in vitro* activities to their appropriate *in vivo* roles.

Divided into a separate domain of life in 1990, archaea have characteristics that are both eubacterial and eukaryotic in nature[37]. Like bacteria, archaea have circular genomes containing genes that are often organized into operons and resemble bacteria in their cell morphologies. In contrast, the archaeal cell cycle and DNA metabolism, such as replication, recombination, repair, transcription, and translation, are reminiscent of eukaryotes[38]. A precedent has already been set for archaea as excellent models for studying eukaryotic systems. Archaeal crystal structures have been particularly useful in helping elucidate

mammalian homologues ranging from signal recognition particles to polymerases[39,40]. In regard to DNA repair, structures of Rad51/BRCA2 interactions and the dual tethering/nuclease function of the Mre11/Rad50 complex have also allowed leaps forward in current understanding of HR[41,42]. Not only have archaeal structures been useful, sequence analysis of *Sulfolobus shibatae* topoisomerase II and comparison with eukaryotic homologues led to identification of Spo11 as the source of DSBs in meiotic HR[43].

Because archaea and eukaryotes handle DNA repair and recombination in a more analogous way than bacteria and eukaryotes, using archaea to model eukaryotic NHEJ is a logical choice. Specifically, the crenarchaeote *Sulfolobus solfataricus* serves as an excellent model[38]. *S. solfataricus* is thermoacidophile that is a convenient archaeon to study for many reasons. *S. solfataricus* is aerobic, unlike many archaea, and can be readily grown in the laboratory on plates or in liquid media[44]. Its approximately 3 Mbp genome has been completely sequenced, and viruses and plasmids from this and similar species have been identified which aid in genetic studies[45,46]. *Solfolobus* species are also attractive models due to the breadth of knowledge currently known about them. Genome wide microarrays are available for these microbes as well as proteomic analyses[47,48].

Additional findings in *S. solfataricus* and other *Sulfolobus* species show them to be particularly eukaryotic-like. First, the attachment of newly replicated chromosomes in G2 phase is prolonged, similar to sister chromatid attachment in eukaryotic replication[49]. This association contrasts with the immediate separation of copied regions in bacterial replication. Secondly, *S. solfataricus*' circular genome contains multiple origins of replication, similar to those found in the linear chromosomes of prokaryotes[50]. Bacterial replication, on the other hand, typically proceeds from a single origin of replication. Finally, *S. solfataricus* contains the only known prokaryotic homologue of eukaryotic Rad54, a key chromatin remodeling protein involved in eukaryotic HR[51,52]. Further studies of this Rad54 protein and its role in archaeal HR will likely give insight into eukaryotic HR.

NHEJ has not been characterized in the domain *Archaea.* However, identification of a putative Ku core located adjacent to a ligase homologue in *Achaeoglobus fulgidus* suggests that NHEJ is a pathway common to all three domains of life[11,12]. Archaea also encode genes for Mre11 and Rad50, two proteins that are utilized in the *S. cerevisiae* NHEJ pathway[41]. The exact role of these proteins in yeast end-joining is unclear, but the complex they form appears to tether broken ends together and may have a similar function in archaeal end-joining[41,53,54]. An end-joining pathway would potentially be very useful to archaea as some of its members grow in DNA-damaging environments, such as temperatures exceeding 100°C[55]. While the lengthy process of HR would ensure full recovery of lost genetic information, the accelerated process of NHEJ may prove more expedient. Archaea have pre-established similarities to eukaryotes in DNA recombination[51]. They also have use for a constant "quick fix" DNA repair method because they naturally exist in DNA damaging conditions; bacteria, in contrast, appear to predominately use NHEJ when stressed[56-58]. If *S. solfataricus* uses this pathway, NHEJ in this archaeon would provide an excellent model for studying the basic system.

In this study we identify an uncharacterized open reading frame (ORF) located in operon-like arrangement with a previously identified DNA ligase homologue and we hypothesize that this gene is a putative *S. solfataricus* Ku[45]. BLAST searches revealed homologues of putative SsoKu in related species, and multiple sequence alignment analysis indicates similarities between this gene and bacterial and eukaryotic Ku amino acid sequences. Real-time PCR results of putative SsoKu and ligase following exposure to ionizing and UV radiation show transcriptional upregulation of these genes following both kinds of damage, implicating that the encoded protein products play a role in DNA repair. *S. solfataricus* whole cell extracts were also assayed for end-joining capability. Joining of linear DNA substrates was consistently found in intact extracts but was reduced to background levels in proteolytically digested samples. Plasmids were isolated from *S. solfataricus* transformed with linearized substrates, but attempts to characterize the method of recircularization of the plasmid were unsuccessful.

3.2 Materials and Methods

S. solfataricus strains. *S. solfataricus* 98/2 and PBL2025 were obtained as a kind gift from P. Blum, University of Nebraska Lincoln, Beadle Center for Genetics. P2 (DSM 1617) was purchased directly from the American Type Culture Collection; it was originally submitted by W. Zillig who isolated it in Italy.

RNA isolation and cDNA preparation for analysis of Ku and DNA ligase transcripts. 10 ml samples were removed from cultures at the times indicated. Cells were pelleted by centrifugation at 10,500 x g. RNA was then isolated using the RiboPure-Bacteria kit (Ambion) and the manufacturer's protocol. Contaminating DNA was removed from RNA samples using the DNA-Free kit (Ambion) following the manufacturer's protocol. cDNA was prepared from DNA-free RNA using the RevertAid First Strand cDNA Synthesis Kit (Fermentas) with random hexamer primers and the manufacturer's recommendations. Resulting nucleic acid samples were quantified at 260 nm using a Coulter Beckman DU-800 Spectrophotometer. Experiments were performed in triplicate from either one or two RNA isolations from each strain.

In vitro assay substrate preparation. A pBluescript T-tailed vector containing the LacS gene from *S. solfataricus* with added *EcoRV* restriction sites at the 5' and 3' ends was digested with *EcoRV* in NEB buffer 3 + BSA (for blunt ended substrates) or double digested with *MscI* and *BamHI* in NEB buffer 4 + BSA (for Blunt – 5' overhang substrates); alternately, pET24a was digested with *XmnI* in NEB buffer 2 + BSA (for blunt-ended substrates). Digested DNA was run on 1% 1 x TBE or 1 x TAE low melt agarose gels and purified as previously described, however glycogen was not added as a carrier molecule[59]. Final concentration was determined by electrophoresing isolated substrates on 0.8% agarose gels, staining with ethidium bromide, and quantifying using the Gene Tools program by Syngene.

Preparation of *S. solfataricus* whole cell extracts. *S. solfataricus* strain 98/2 was grown in rich medium containing 0.2% (wt/vol) sucrose, 0.2% (wt/vol) tryptone, and basal salts (ST medium) to a desired optical density, measured by absorbance at 540 nm. Cultures were centrifuged at 8.5 krpm for 15 min using an SA-600 rotor in a Sorvall SC-5B Plus centrifuge. After removing the supernatant, cells were resuspended in 25 mL of growth medium, re-centrifuged, and finally resuspended in 2 mL of a buffer containing 200 mM sodium acetate, pH 6.0, 1mM DTT, and 11% glycerol. Cells were disrupted by sonication using a Branson sonifier 250. Sonicates were ultracentrifuged in Beckman open top thickwalled polyallomer tubes for 1 hour at 60,000 rpm in a Beckman Optima TLX Ultracentrifuge with a TLA100.4 rotor. The cell-free extract was collected and dialyzed against resuspension buffer. Absorbance measurements were determined at 260, 280, and 350 nm to estimate the abundance of DNA, protein, and aggregates in the cell-free extracts. Total protein concentration was calculated using the absorbance measurement at 280 nm, and the extracts were flash frozen and stored for future use at -80°C.

In vitro end-joining activity assay. Approximately 55 ng of protein in whole cell extracts were added to a mixture of 400 ng DNA substrate and reaction buffer (to a final concentration of 18 mM MES, pH 6.3 at 25°C, 22 mM $Mg(OAc)_2$, 4 mM ATP, 1 mM DTT) to a final volume of approximately 200 µL. Reactions were incubated for one hour at 70°C in a PCR machine with a hot lid set to 90°C. After the reaction, samples were extracted twice with 1:1 phenol-chloroform and once with 1:50 chloroform-isoamyl alcohol. They were then ethanol precipitated with or without the use of glycogen as a carrier agent. DNA was resuspended in TE buffer, electrophoresed on 0.8% agarose gels, and stained with SYBR green. The same manipulations were performed with negative control experiments that contained extract buffer instead of whole cell extracts. As an alternate negative control, extracts were pre-digested with Protease S by incubating 0.2 U Protease S with 55ng extract protein at 95 °C for 30 min; the assay was then performed

with these degraded extracts. Image contrast adjustment was performed using Microsoft Office image editing.

In vivo assay substrate preparation. pJlacS was digested with *XhoI* in NEB buffer 2 + BSA, then heated at minimally 65°C for 20 minutes to inactivate the restriction enzyme[60]. For substrates with compatible 5' overhangs, the digest was electrophoresed directly on a 1xTBE agarose gel. For substrates with blunt ends, dNTPs were added to the heat treated digest to a final concentration of 33 µM. Approximately 2 units of Klenow fragment per microgram of substrate was added, and reactions were incubated at 10°C for 15 min and then stopped with the addition of 0.5 M EDTA. Reaction were then electrophoresed on 0.6-0.8% low-melt agarose gels and purified[59]. For substrates with incompatible 5' overhangs, d(CT)TPs were added to the heat treated digest to a final concentration of 1mM, followed by the addition of approximately 1 U Klenow per microgram of DNA substrate. The reaction was incubated at 37°C for 1 hour, then extracted twice with 1:1 phenol:chloroform and once with 1:50 chroroform:isoamyl alcohol. The substrate was then ethanol precipitated using ammonium acetate as the cation source. Precipitated DNA was resuspended in 16 µL H_2O, 2 µL T4 ligase buffer, and 2 µL T4 ligase. The reaction was incubated at 16°C overnight and electrophoresed on an agarose gel for purification. Substrates were gel purified from low melt agarose gels or using a Qiagen gel purification kit according to manufacturer's recommendations[59].

In vivo assay to test for the recovery of transformed plasmids. *S. solfataricus* strain PBL2025 was grown in 0.2% ST medium to mid-exponential phase (approximately an OD_{540} of 0.2 – 0.4) and cells were collected by centrifugation at 8.5 krpm for 15 min using an SA-600 rotor in a Sorvall SC-5B Plus centrifuge. Cells were washed by resuspending them in 25-30 mL of cold 20 mM sucrose. Following a second wash in approximately 10 mL of cold sucrose, the cells were resuspended to a final volume of approximately 250 µL of cold sucrose. 46 – 56 ng of each DNA substrate, including an uncut, supercoiled pJlacS positive control,

was incubated with 50 µL of concentrated cells on ice for 20 min. The cells were transformed by electroporation at 1500 V, 400 ohms, and 25 µF in 1 mM cuvettes. Electroporated cells were immediately resuspended in 1 mL sterile water and incubated on ice for 1.5 – 3 min. The cells were then moved to a 72 – 77 °C water bath and incubated for 10 min before adding them to prewarmed 0.4% lactose basal salts medium. The optical density of the cultures was monitored at 540 nm until the cells reached early to late logarithmic growth phases. The cells were then collected by centrifugation at 8.5 krpm for 15 minutes using an SA-600 rotor in a Sorvall SC-5B Plus centrifuge and plasmids were isolated using either a Qiagen Miniprep Kit (according to manufacturer's suggestions), a Wizard Plus Miniprep kit (according to the manufacturer's suggestions), or the alkaline lysis method. In this method, were resuspended in 100 µL of sterile TEG (25 mM Tris-Cl, pH 8.0, 10 mM EDTA, 50 mM glucose) buffer and incubated at room temperature for five minutes. Next, 200 µL of 0.2 N NaOH + 1% SDS was added to each tube, mixed gently, and incubated at room temperature for five minutes before adding 150 µL of cold potassium acetate. Samples were then mixed gently, incubated on ice for five minutes, and centrifuged for five minutes at 13.3 kprm in a tabletop Labnet Spectrafuge 24D centrifuge. Supernatants were removed, extracted once with 1:1 phenol/chloroform once with 50:1 chloroform/isoamyl alcohol, and finally ethanol precipitated. Recovered plasmids were digested with *XhoI* in NEB buffer 2 along with a supercoiled pJlacS positive control and electrophoresed on a 0.8% 1 x TBE agarose gel for analysis.

3.3 Results

3.3.1 Identification of a putative SsoKu ORF.

The first implication of NHEJ in archaea was reported in 2001 when a putative Ku and ATP dependent DNA ligase were identified in the euryarchaeote *Archaeoglobus fulgidus* [12]. *S. solfataricus* has one DNA ligase (SsoLigase) that has been previously reported and characterized[61,62]. Sequence comparisons reveal that SsoLigase bears 31% and 33% identity to *S. cerevisiae* ligase IV and the putative *A. fulgidus* ADDL, respectively, using the Blosum62 scoring matrix of the BestFit comparison program. Upon investigation of its genomic environment, it was noted that a small, uncharacterized open reading frame is situated just upstream of SsoLigase; the proximity of this second ORF (SSO0188) suggests that the two genes are arranged in an operon. Archaea, like bacteria, arrange genes that are involved in similar pathways in operons. Therefore, if the putative ligase is indeed involved in an NHEJ-like pathway, this second ORF is highly likely to be involved in it as well. I hypothesize that this gene is an archaeal Ku. This gene is conserved among other *Sulfolobales* and closely related crenarchaea (BLAST results indicated 50% (*Sulfolobus acidocaldarius* Saci0787), 55% (*Sulfolobus tokodaii* ST0222), 55% (*Metalosphera sedula* Msed0149), identities with the *S. solfataricus* SSO0188 amino acid sequence), implying that it has an important function. Putative SsoKu and its *S. tokodaii* homologue were compared with eukaryotic Ku70 (*H. sapiens* and *S. cerevisiae*), bacterial Ku (*M. tuberculosis* and *P. aeruginosa*), and the putative Ku in *A. fulgidus* using the MUSCLE multiple sequence alignment algorithm[63]. Seventy-nine similar residues (determined by the Blosum62 scoring matrix) were found to be conserved in 5/7 or more of the compared organisms (Figure 3.1)[64]. Both *Sulfolobales* shared 75% of these conserved residues, suggesting these uncharacterized ORFs may indeed be Ku homologues.

3.3.2 Real Time PCR (RT-PCR) analysis of putative SsoKu and DNA ligase transcripts following DNA damage.

To assess whether transcription of the DNA ligase and hypothetical SsoKu ORF is responsive to DNA damage, exponentially growing *S. solfataricus* cultures were exposed to 250 Grays of ^{60}Co radiation or 100 J/m^2 ultraviolet radiation, and returned to normal culture conditions. Both exposures are sublethal doses of radiation, resulting in approximately 30-40% survival after gamma and 10-20% survival after UV irradiation. All samples were treated similarly after irradiation, however samples exposed to UV were kept in the dark to inhibit light repair of thymine dimers[65,66]. RT-PCR was performed to quantify the number of putative SsoKu and SsoLigase transcripts relative to 23S rRNA transcripts, which are constitutively produced and thus an effective control[67].

Results indicate that *S. solfataricus* both DNA ligase and putative Ku transcripts increase in abundance following ionizing and UV radiation (Figure 3.2). Transcript levels in *S. solfataricus* P2 were tightly coupled: forty-five minutes after gamma irradiation, putative SsoKu and Ligase transcripts increased approximately twenty and forty fold, respectively, but they increased relatively little at other time points. Likewise, thirty minutes after UV irradiation, ligase and putative SsoKu transcripts were present at approximately ten and twenty fold over untreated levels, respectively, but transcript levels changed very little at other time points. The response of *S. solfataricus* 98/2 was distinct. For both genes, two waves of transcription were observed at thirty and sixty minutes after ionizing radiation; during these waves, putative SsoKu transcripts increased approximately 2- 4.5 fold and DNA ligase transcripts reached approximately 4.5 – 6 fold over constitutive levels. Similar patterns were observed following UV radiation, however lower transcript levels were observed. A single, extended wave of upregulation was seen from thirty to sixty minutes and peaking at forty-five minutes with approximately 1.5 and 2 fold levels of SsoKu and ligase transcripts, respectively. Despite their operon-like arrangement, putative SsoKu and ligase transcript levels varied relative to each other, however, in most cases they stayed within 2-3 fold of each other.

Overall, the RT-PCR data supported our hypothesis that the proteins encoded by the putative SsoKu and SsoLigase are involved in DNA damage repair. It is logical that transcripts of proteins involved in the repair of a certain kind of damage (here, DSBs) would increase in abundance in response to that damage.

3.3.3 Development of an in vitro *cell free extract assay.*

Diggle, *et al.* report the use of a small-scale cell free extracts assay to detect NHEJ in human cell cultures that was adapted from a previous protocol[68,69]. I have modified this assay for use with archaeal extracts to detect end-joining. For these experiments, cell extracts were obtained by sonication and ultracentrifugation as described in the Materials and Methods. Briefly, to initiate an assay, extracts were added to a buffered reaction mixture containing linearized dsDNA. Negative controls were performed in the same manner except extracts were substituted with buffer, or extracts that had been proteolytically degraded with a thermophilic protease were substituted with intact extracts. Assays were incubated at 70°C for one hour, and the resulting products were visualized on agarose gels stained with Sybr Green.

Figure 3.3A depicts expected positive and negative results of the *in vitro* assy. If end-joining occurs, the plasmid will be circularized or multimerized (i.e. by joining multiple copies of the plasmid together) and be manifested as additional bands. These DNA products will display reduced mobility indicative of either relaxed circular DNA (if the plasmid ends were rejoined) or larger pieces of DNA (if multiple substrates were ligated together) as compared to the linear input plasmid DNA. Figure 3.3B-D shows results acquired from this assay. The band shifts seen in the experimental but not negative control lanes clearly demonstrate that the substrate is being ligated either to itself or other substrates and that end-joining is occurring. The negative control performed with Protease S-degraded extracts demonstrated the need for intact protein to obtain a joined product rather than a mix of peptides.

3.3.4 Characterization of end joining with an in vivo *plasmid repair assay.*

Berkner*, et al.* report a series of *E. coli - Sulfolobus* shuttle vectors that are able to autonomously replicate in both organisms[60]. One of these vectors, pJlacS, harbors the *Sulfolobus* LacS gene and pyrEF operon; the former encodes the beta-glycosidase enzyme and the latter encodes two enzymes that are crucial in the *Sulfolobus* uracil biosynthesis pathway. Uptake of this plasmid yields viability selection in ΔlacS and/or ΔpyrEF strains. The vector also contains the *bla* gene for ampicillin resistance in *E. coli*.

To test the ability of *S. solfataricus* to perform end-joining *in vivo*, linearized pJlacS was transformed into a ΔlacS strain (*S. solfataricus* PBL2025) and plated on a selective medium containing lactose as the sole carbon source. Transformed substrates had compatible 5' overhangs, incompatible 5' overhangs, or blunt ends. In addition, an uncut positive control was also used. All transformed cultures began to grow after six days and there was no appreciable difference in the time required for the different cultures to return to exponential growth (data not shown). Plasmids were then isolated from these culture and evaluated by electrophoresis (Figure 3.4). The plasmids extracted from all cultures displayed approximately the same molecular weight and were larger than pJlacS amplified from *E. coli*. Because *XhoI* cleaves pJlacS at a single location, isolated plasmids from the transformed cultures were digested with *XhoI*, however the ability of the enzyme to cleave the isolated plasmids was unclear. Attempts to sequence across the hypothesized joined regions were unsuccessful. There are several reasons why this may have occurred. First, an extensive region of resection may have provided no DNA template for the primers. Alternatively, a major product may not have been obtained if multiple plasmids of undetectable size differences were present due to multiple processing events. Due to the large size of the isolated plasmids, we also cannot rule out recombination of the plasmid with the genome at the *pyrEF* locus. As an alternative approach for assessing end-joining, and to analyze individual end-joining events, cultures were plated on lactose medium directly after the transformation process in order to isolate single colonies. While colonies were obtained, attempts to sequence across the hypothesized joined region were unsuccessful

here as well. Colonies were also found on plates from control experiments in which non-transformed control cells were grown on lactose medium, thus the stringency of the selection system appears insufficient.

3.4 Discussion

Simple NHEJ pathways have been identified in bacteria, and many contain Ku and DNA ligase genes in close proximity or in operon arrangement. A putative Ku has been reported in the euryarchaeon *A. fulgidus* but has not been further characterized beyond this initial identification[12]. A gene immediately upstream of the *S. solfataricus* DNA ligase homologue that has similarity to both eukaryotic and bacterial Kus was identified and is a promising candidate for an archaeal Ku. The presence of homologues in related species supports the hypothesis that this gene plays a non-trivial role in these organisms.

The observed upregulation of putative SsoKu and SsoLigase transcripts in response to DNA damage is a promising indicator that these genes are involved in DNA repair. We expect upregulation of these transcripts in response to gamma radiation if their encoded proteins are indeed involved in DSB repair, since IR produces DSBs (see Chapter 1). Thus, the observed upregulation of putative SsoKu and SsoLigase in both strains supported a role for these proteins in DSB repair. While UV radiation is not typically considered a source of DSBs, accumulation of these breaks has been reported to occur after irradiation and may be due to errors in replication and repair[70,71]. Therefore, DSB repair proteins are not expected to be as strongly upregulated in response to UV as they are to IR, as was observed.

By optimizing a simple assay designed to test for NHEJ in mammalian cells, I found consistent end-joining activity in *S. solfataricus* whole cell extracts[68,69]. The presence of a band migrating at a higher molecular weight than the linear substrate indicated that either the linear substrate was circularized, multimerized, or both. Joining occurred with blunt ended substrates as well as substrates with both blunt and 5' overhanging ends, however it is not known how the latter substrates were joined because multimerization may have occurred between compatible ends. The lack of joining activity in the Protease S digested control indicates that this activity is an intact protein-specific event instead of one catalyzed by peptide fragments. The source of this end-joining activity is not yet known – it could be due to a simple DNA ligase or it may be due to a previously undescribed archaeal end-joining pathway. Since we have

identified a hypothetical SsoKu gene in operon-like arrangement with SsoLigase homologue, we suggest the latter.

To determine whether this activity could be detected *in vivo*, a second assay was modified for use in archaea. The *in vivo* plasmid repair assay has been utilized to study the role of different NHEJ factors in both eukaryotic and eubacterial systems and has also been used to assess NHEJ in patients with genetic defects in order to better understand how this pathway is affected[72-74]. The *in vivo* religation of a plasmid with no homology to the host chromosome is indisputable evidence of the presence of an end-joining pathway, making this assay is a valuable tool for detection and analysis of NHEJ. Preliminary results obtained for this approach were equivocal. Although plasmids were recovered after linear substrates were transformed into *S. solfataricus*, the nature of these plasmids is not well understood since they were larger than the transformed substrate and attempts to sequence across the joined region were unsuccessful. Further development and optimization of this assay will not only help confirm whether or not end-joining occurs in *S. solfataricus* but also give mechanistic insights into pathway by allowing analysis of fidelity of plasmid repair.

3.5 Acknowledgements

Dr. Michael Rolfsmeier performed the Real Time PCR experiments described in this work.

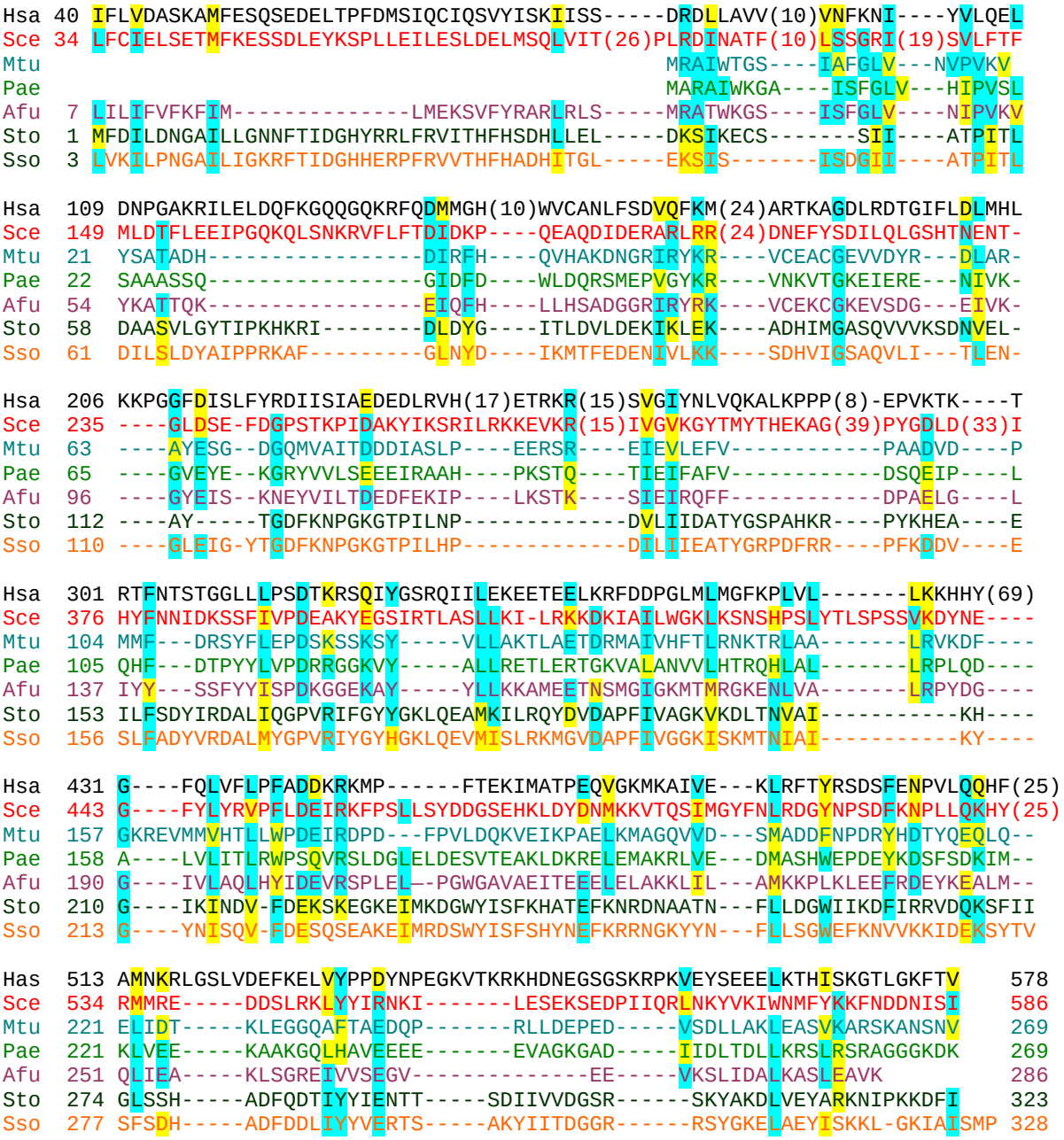

Figure 3.1 Multiple sequence alignment of eukaryotic, bacterial, and putative archaeal Kus. Sequence alignment of bacterial and eukaryotic Ku homologues alongside putative archaeal Kus was performed using MUSCLE multiple sequence alignment algorithm. Using the BLOSUM62 scoring matrix, residues were highlighted that were similar in 5/7 of the aligned sequences (blue highlighting indicates identical residues and yellow highlighting indicates similar residues). HsKu70, *Homo sapiens* Ku70; SceKu70,

Saccharomyces cerevisiae Ku70; Mtu, *Mycobacterium tuberculosis* Ku homologue (Rv0937c); Pae, *Pseudomonas aeruginosa* Ku homologue (PA2150); Afu, *Archaeoglobus fulgidus* putative Ku homologue (AF1726); Sto, *Sulfolobus tokodaii* putative Ku homologue (ST0222); Sso, *Sulfolobus solfataricus* putative Ku homologue (SSO0188).

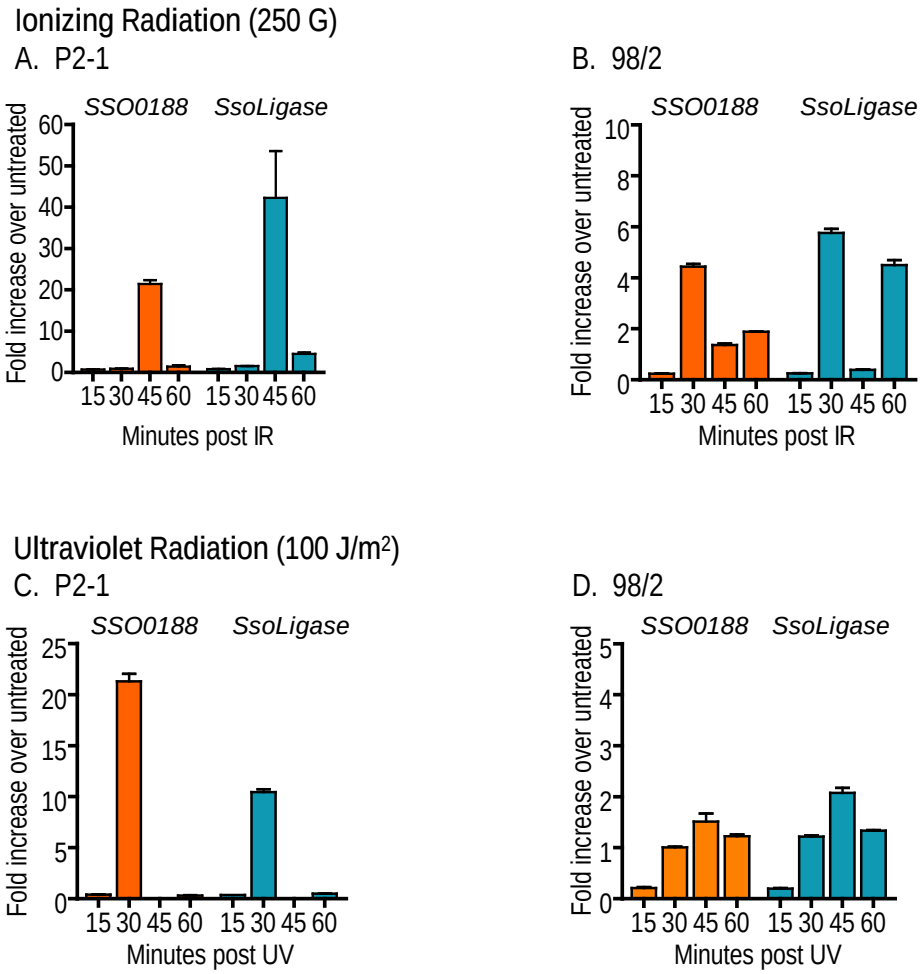

Figure 3.2 Putative SsoKu and SsoLigase mRNAs are upregulated in response to DNA damage. Preliminary Real Time PCR results of putative *S. solfataricus* SsoKu and DNA ligase transcript regulation following gamma irradiation (A, B) and UV irradiation (C,D).

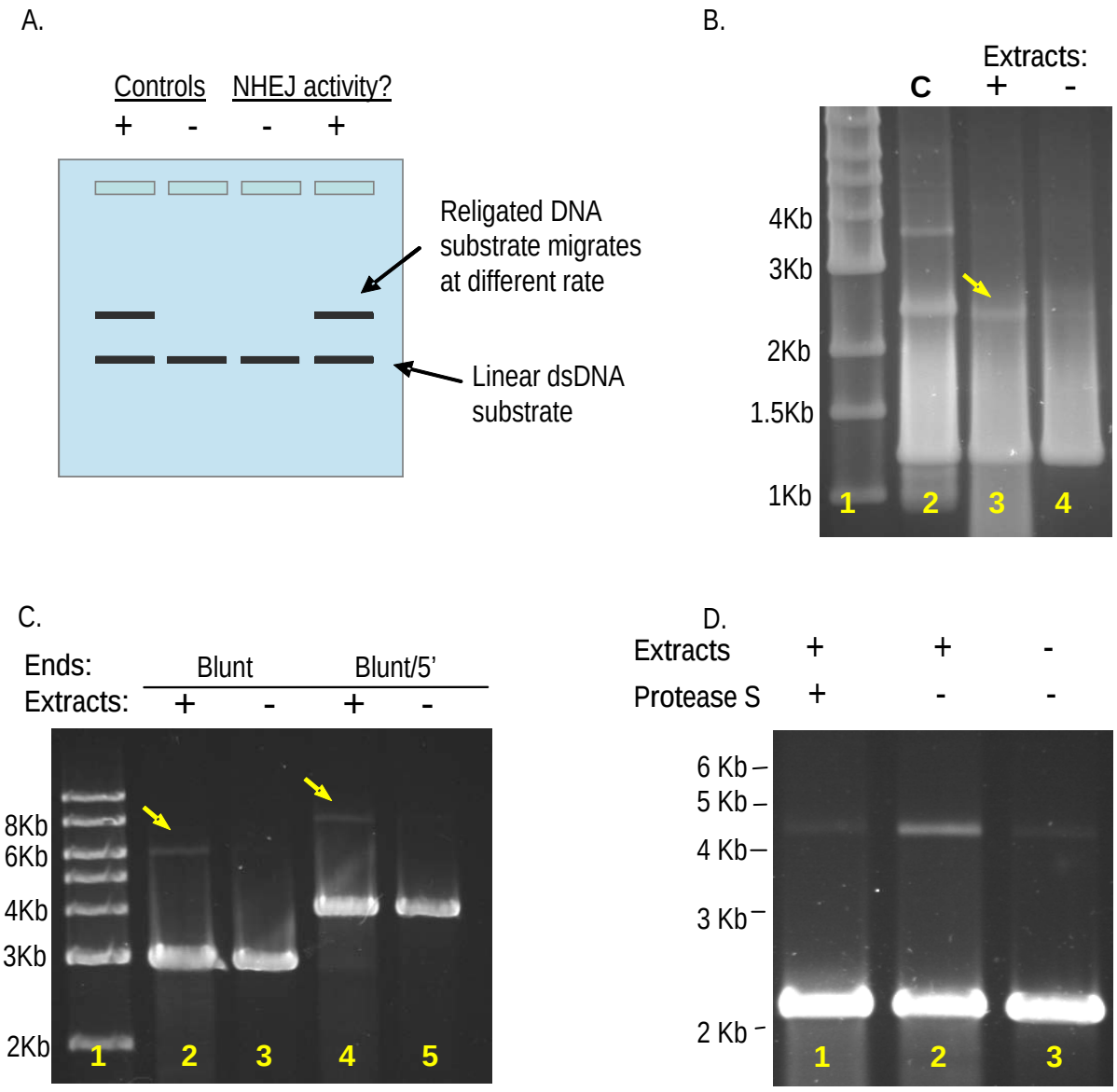

Figure 3.3 Development of an *in vitro* assay to test for end-joining in *S. solfataricus*. **A.** Schematic of hypothetical results that could be obtained from the *in vitro* whole cell extracts assay. The positive control will have one or more bands migrating at a higher molecular weight than the linear substrate due to circularization or multimerization by joining DNA ends. No higher molecular weight bands will be observed in the negative control. If end joining occurs in the whole cell extracts, a band corresponding to the circularized or multimerized product from the positive control will be visible. **B.** Whole cell extract assay results using blunt and blunt/5' overhanging ended dsDNA substrates. Lane 1, 1 Kb ladder. Lanes 2, 4

whole cell extracts added to reaction. Lanes 3, 5 negative controls (buffer substituted for extracts). C. Assay with blunt ended dsDNA substrate. Lane 1, 1 Kb ladder. Lane 2, T4 ligase ligated substrate control. Lane 3, whole cell extracts added to reaction. Lane 4, negative control (buffer substituted for extracts). D. Whole cell extract assay with blunt ended dsDNA substrate. Lane 1, Protease S degraded extract control. Lane 2, reaction with whole cell extracts. Lane 3, negative control (buffer substituted for extracts).

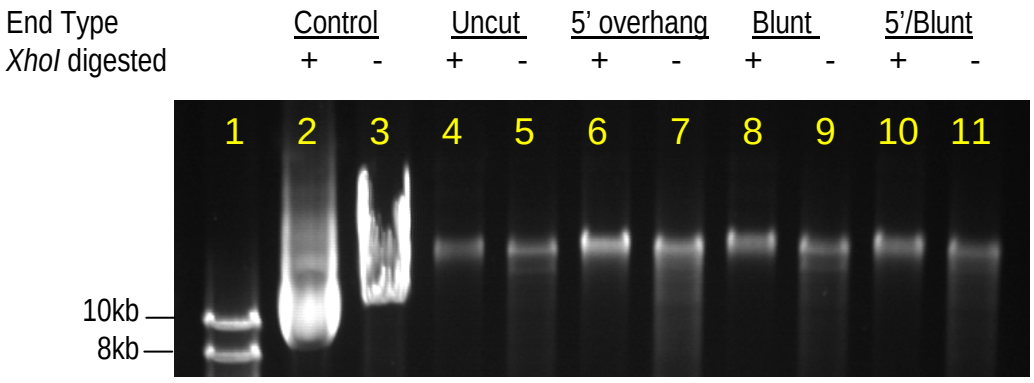

Figure 3.4 Plasmids can be recovered from *S. solfataricus* transformed with linearized pJlacS. Lanes 2, 3, untransformed pJlacS (for size comparison). Transformed substrates were uncut pJlacS (lanes 4,5), linear plasmids with 5' compatible overhangs (lanes 6,7), linear plasmids with blunt ends (lanes 8,9), and linear plasmids with noncomplementary 5' overhanging/blunt ends (lanes 10, 11).

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CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

The work presented in this thesis improves current knowledge concerning the response of *S. solfataricus* to radiation and the factors it uses to repair this damage. The progress that has been made in understanding *Sulfolobus'* damage repair mechanism is four fold. First, this is the first comparison of strain dependent repair to UV and IR damage. Second, this is the first substantiated report of the increase in *radA* and *rad54* transcripts in response to UV and ionizing radiation. Third, this is the first report that the *rad54* gene varies in size in different *S. solfataricus* strains and that a protein product is only produced to detectable levels when it is transcribed from a full length gene. Fourth, this work gives the first cell-based evidence for an archaeal end-joining pathway. This chapter summarizes each of these findings and discusses future directions that will stem from each discovery.

4.1 Strain dependent response to damage

S. solfataricus has been isolated from multiple geographical locations and genome analysis indicates distinct strain dependent features. One of these features is the presence of large numbers of insertion (IS) elements in P2 genome that are less prevalent in the 98/2 genome (i.e. 11% versus 5%, respectively)[1,2]. The effect that these IS elements has on the physiology of P2 is not well understood, but is likely significant. In Chapter 2, I report consistent differences between the abilities of strains P2 and 98/2 to recover from ionizing and UV radiation. One specific difference is that the P2 strains recover more quickly to high UV doses than strain 98/2. Whether the differences in recovery are due to the presence of IS elements is uncertain. Due to their tighter regulation of *radA* and *rad54* transcription in response to UV damage, we hypothesize that P2 strains maintain tighter control of their genome. IS element hopping can be a dangerous event, especially if an essential gene is disrupted, and HR-mediated transposition is a well

known occurrence[3]. With so many IS elements present in its genome, P2 may very carefully regulate the expression of HR proteins in effort to protect its genome. This tight regulation may not be as necessary in 98/2 because it has significantly fewer IS elements, resulting in an extended period of heightened transcript abundance opposed to punctuated increases in expression that quickly diminished. This does not, however, explain the strain dependent difference in recovery times.

The observed differences in the recovery of the P2 derivatives from strain 98/2 may be in part due to differences in the ability of these strains to perform DSB repair and/or their preferred method of repair. Specifically, strain 98/2 may prefer to repair DNA damage via the HR pathway while the P2 derivatives may favor other methods of repair, such as nonhomologous end-joining (see Chapter 3). A method of targeted gene disruption has been reported and optimized using a stable *lacS* mutant of strain 98/2[4,5]. In this method, a recombinagenic substrate is constructed by cloning the gene of interest into an *E. coli* vector and disrupting it with the *lacS* gene. This substrate is transformed into the mutant 98/2 strain and recombination of the transformed disrupted gene with the original gene is forced via lactose selection[4,5]. Interestingly, reports of successful gene disruption are exclusively limited to strain 98/2. This may support a hypothesis that strain 98/2 either prefers HR as a method of DSB repair or that it has a more efficient HR pathway than the P2 derivatives. Analysis of the abundance of transcripts of genes involved in the *S. solfataricus* HR and putative end-joining pathways in these strains does not directly indicate strain favoritism for a particular pathway, as the levels of *radA* following IR do not differ significantly from the levels of putative SsoKu and/or SsoLigase transcripts for each strain. Nonetheless, strain 98/2 is potentially able to respond to damage faster than strain P2, as transcript abundance for all genes assessed peaks around about fifteen minutes earlier in this strain. Future studies of HR in these strains, including studies of the kinetics of DSB repair, will elucidate some of these unresolved issues.

4.2 Differential transcript abundance of HR-related genes following UV and ionizing radiation.

Our findings that *radA* transcripts increase in response to UV damage seemingly conflict with previous findings in *S. solfataricus*, but may be due simply to strain-specific differences or differences in procedures followed (see Chapter 2 Discussion)[6-8]. This is the first report of *radA* transcript levels following IR and the first report of *rad54* transcript levels following any form of DNA damage. To confirm our results, western hybridization analysis should be performed to support transcript findings. Preliminary studies are currently underway to determine SsoRadA and SsoRad54 protein levels over a time course following UV damage and they will be followed by analyses of protein levels following IR. We hypothesize that SsoRadA and SsoRad54 protein levels will increase following radiation damage consistent with their transcriptional profile. We realize, however, that this effect may be difficult to accurately quantify due to the fact that a majority of cells (about 80-90% of cells following UV and about 60-70% following IR) die following the chosen radiation doses. However, the chosen UV dose, which results more cell death than the chosen IR dose, is similar, if not lower, than that used for other UV studies with this organism[6-8]. Increases in *rad54* and *rad51* transcript levels have previously been reported in yeast[9-12]. Studies indicate that *S. solfataricus* has a eukaryotic-like HR pathway, thus our findings of similarities in expression patterns of HR-related genes following DNA damage are not surprising.

The increased abundance of transcripts following DNA damage supports a role for their encoded protein products in mending it. Future studies of the archaeal Rad54 homologue will include investigations of its *in vivo* role in HR. Initially, co-immunoprecipitations can be performed to determine which proteins SsoRad54 interacts with in the cell. *In vitro* studies demonstrate direct SsoRadA-SsoRad54 interactions, and these should be confirmed *in vivo*[13]. Secondly, the Haseltine lab is in the process of developing an archaeal system for inducing isolated double-strand breaks in a known location of the genome. Once this system has been developed, the kinetics of DNA repair can determined as well as the kinetics of RadA protein and Rad54 protein arrival at a double-strand break site. Studies of eukaryotic HR proteins are

complicated by the fact that many have multiple cellular functions. Findings in archaea may provide insights into complicated eukaryotic pathways due to their relative simplicity.

4.3 Size variation of the *rad54* gene and abundance of protein product

This is the first report of natural mutations in a *rad54* gene leading to a loss of protein product. While it well known that *rad54* is not essential for viability, the breadth of *in vitro* activities that this protein has which implicate roles spanning from presynapsis to postsynapsis make it unclear why such mutations would not be selected against. Although missense mutations have been found in cancer patients, it is important to emphasize that natural mutations that would lead to truncations of a translated protein product, such as those found in strains P2-2 and 98/2, have not been reported in eukaryotic organisms[14]. While eukaryotes carry Rad54 homologues that appear to have roles in meiotic recombination, a loss of function mutant in archaea would appear to be more severely disadvantaged due to apparent lack of any SsoRad54 homologues with overlapping activities[15]. Several experiments could be done to clarify the results shown in Chapter 2. To determine if SsoRad54 is produced at levels that are below the normal detection capabilities of the anti-Rad54 antibody in strains P2-2 and 98/2, co-immunoprecipitations could be performed with RadA protein. *In vitro* studies indicate that SsoRadA and SsoRad54 directly interact[13]. Using RadA to fish for Rad54 in whole cell extracts could be an effective way of reducing background on western hybridizations and effectively concentrating low levels of Rad54 truncated products to detectable levels. Control reactions would be performed using heterologously expressed protein to ensure that failure to detect a product is not due to altered RadA-Rad54 interactions. Eukaryotic Rad54-Rad51 interactions occur within the N-terminal domain of the Rad54 protein[16,17]. It is hypothesized that that this interaction will be conserved in the archaeal homologues, thus RadA-Rad54 interactions should be maintained in experiments with truncates. If a truncated protein product is detected, albeit at lower levels, the activity of

the truncated protein will be analyzed. This could be accomplished via heterologous expression and purification of the truncated protein from *E. coli* and assaying its activity.

To clarify whether loss of Rad54 protein leads to a repair-deficient phenotype, expression of full length SsoRad54 could be induced in strain PBL2025 using the expression system described above. Strain PBL2025, like strain 98/2, encodes a *rad54* gene with a short insertion that would lead to loss of three of the protein's seven conserved helicase domains in the translated protein product. The transformed strain would be tested for recovery from DNA damaging agents such as gamma or UV radiation to determine whether the full length protein product assists in the cells' recovery to damage. A mechanism of knocking out genes in strain P2-1 has not been reported, however one may become available in the future. When one emerges, an alternative approach would be to delete the full length protein and assay the strain for increased sensitivity to DSBs.

Due to the fact thermophilic enzymes are often most active near their host's growing temperatures (i.e. often 80°C or higher) and have little activity near the growing temperatures of mesophiles such as *E. coli* and *S. cerevisiae*, it is unclear whether many archaeal HR proteins can work together with factors from homologous pathways to mediate HR processes, although SSB has been reported to stimulate the strand exchange activity of *E. coli* RecA[18]. However, if *Sulfolobus* based methods prove difficult, the effect of a truncated Rad54 protein could be studied in yeast. By overexpressing yeast Rad54 proteins with truncations equivalent to those of translated *S. solfataricus rad54* inserted genes in *rad54* deleted backgrounds, sensitivity assays could be used to clarify whether the truncates confer any resistance to DSBs.

4.4 Investigation of a potential archaeal end-joining pathway

A putative Ku homologue was identified over eight years ago in the euryarchaeon *Archaeoglobus fulgidus*, however no further studies of an end-joining pathway in archaea since that time[19,20]. In Chapter 3,

we identify a putative Ku homologue in *S. solfataricus* that bears homology to eukaryotic, bacteria, and putative archaeal Kus. We then support the identified gene's involvement in DNA damage repair with RT-PCR data that indicates transcriptional upregulation following radiation. Furthermore, found consistent end-joining activity in *S. solfataricus* extracts. Collectively, this work gives promising evidence for an archaeal end-joining pathway. Future work on this project will have two objectives: the characterization of end-joining in *S. solfataricus in vitro* and *in vivo.* A major goal of *in vitro* studies will be to heterologously express and purify putative SsoKu to determine whether it has similar activities to other known Ku proteins. In particular, the ability of SsoKu to bind DNA and stimulate the joining of linear substrates by heterologously expressed SsoLigase will be investigated. The effect of adding heterologously expressed SsoKu to the *in vitro* end-joining assay will also be analyzed. Preliminary efforts to clone the putative SsoKu ORF into pET, pBAD, and pGEX *E. coli* expression vectors, a pSVA *S. solfataricus* expression vector, and a pPICZ yeast expression vector have been undertaken. All of these vectors rely on amplification of the plasmid in *E. coli* and preliminary results indicate that SsoKu may be toxic to *E. coli,* since all resulting clones were found to be mutated, most often by DNA point mutations but also by larger rearrangements of the SsoKu gene. Because *E. coli* does not utilize an end-joining pathway, we hypothesize that a DNA-binding protein like Ku may cause havoc for its genome. Future work will continue using minimally leaky expression plasmids and/or possibly cloning this gene in yeast. As previously described, NHEJ has been found in yeast but not *E. coli,* therefore it may respond better to heterologously expressed Ku. Additionally, yeast grows at a lower temperature than *E. coli*, which would make heterologously expressed SsoKu less active within the host. *In vivo* studies will also be continued to investigate end-joining in live *S. solfataricus* cells. Characterization of circularized plasmids will not only confirm end-joining of these organisms but also give insights into the pathway's mechanism by assessing the fidelity of the resulting sequence. To accomplish these tasks, a more stringent selection may be required. A recent pyrimidine auxotroph of strain PBL2025 was recently isolated in our laboratory which

may provide this needed selection. One difficulty of working with lactose selection is the inherent breakdown of lactose at high temperatures over time to substrates that can be metabolized by lacS mutants. Such breakdown resulting in an alternative usable substrate does not occur in a pyrimidine auxotroph selection system.

4.5 Summary

In conclusion, the results presented in this thesis advance our current understanding of the response of *S. solfataricus* to radiation damage. Specifically, these findings combine both *in vivo* and *in vitro* studies to broaden our knowledge of *Sulfolobus* double-strand break repair pathways. Future directions derived from these studies will result in promising insights into archaeal HR and end-joining activities and will likely lead to further insights of more complex, yet homologous, eukaryotic systems.

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