The Radioresistance of Deinococcus Radiodurans.

Valerie Mattimore
Louisiana State University and Agricultural & Mechanical College

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THE RADIORESISTANCE
OF
DEINOCOCCUS RADIODURANS

A Dissertation
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Microbiology

by
Valerie Mattimore
B.S., University of Tulsa, 1990
May 1997

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ABSTRACT

*Deinococcus radiodurans* is a remarkably ionizing radiation resistant bacterium. As a vegetative cell it is capable of withstanding 5,000 Gy ionizing radiation without evidence of death or mutation. *D. radiodurans* survives irradiation by repairing DNA damage via an extremely efficient DNA repair system. However, this DNA repair system has not been completely defined. In addition, the selective process allowing the evolution of high level radioresistance has remained a question. This dissertation bolsters the understanding of the DNA repair system of *D. radiodurans* by characterizing a collection of ionizing radiation sensitive (IRS) mutants. The level of radioresistance is quantified and transformation ability is assessed for each IRS mutant. A new subset of mutants slow to recover from irradiation is identified. The IRS mutant collection is organized into sixteen linkage groups. Using linkage group data, the order of mutations within the *pol* linkage group is determined and the distance between cotransformed markers is found to be unusually small. The *irrI* mutant within linkage group I is observed using pulsed-field gel electrophoresis (PFGE) to examine mutant DNA during a post-irradiation recovery time course. This information in conjunction with survival data provides evidence that the *irrI* gene product
inhibits DNA degradation at DNA gaps or double-strand breaks (dsbs). This dissertation also provides evidence which indicates a selection process by which extreme radioresistance mechanisms could have evolved. A correlation is found between desiccation sensitivity and ionizing radiation sensitivity within the IRS mutant collection. PFGE observation of desiccated and ionizing radiation irradiated cell samples demonstrate that the DNA within these cell samples contains multiple dsbs. Because desiccation and ionizing radiation cause a similar pattern of DNA damage in the form of dsbs, the DNA repair mechanism used to survive ionizing radiation exposure could have evolved in response to periods of desiccation.
CHAPTER ONE - INTRODUCTION

*Deinococcus radiodurans* exhibits remarkable resistance to ionizing radiation. As a member of the most radioresistant genus of organisms ever identified, it is approximately 6,000 times more resistant to ionizing radiation than human cells in culture. However, the mechanisms behind *D. radiodurans*’ extreme radioresistance have remained poorly understood.

Early research on the radioresistance of *D. radiodurans* focused upon identifying passive mechanisms of radioresistance. Cellular material was examined for the presence of radioprotective chemicals. It was believed that the cell envelope would contain carotenoids or other chemicals capable of providing extensive radiation resistance, or that cell extracts would contain more efficient radical quenching enzymes. However, passive processes like these have been shown to play only a minor role in radioresistance. Evidence indicates that *D. radiodurans* suffers radiation-induced DNA damage and survives by repairing that damage. Therefore, an extremely efficient DNA repair system is believed to mediate the extraordinary radioresistance exhibited by *D. radiodurans*.

Major questions concerning the radioresistance of *D. radiodurans* have persisted despite forty years of
research. One question concerns the selective processes that allowed extreme radioresistance to evolve in *D. radiodurans*. The radioresistance appears to be the result of an evolutionary process that selected for organisms able to tolerate massive DNA damage. However, radioresistance of the magnitude seen in the deinococci cannot be a direct adaptation to ionizing radiation, because there is no selective advantage to being ionizing radiation resistant in the natural world. No terrestrial environments generate such high levels of ionizing radiation (61). It must, therefore, be assumed that deinococci's radioresistance is an incidental use of the DNA repair capability of this cell.

This dissertation attempts to answer this question by providing evidence that the DNA repair capabilities of *D. radiodurans* result from selection for desiccation resistance.

Another persistent question concerns the nature of the enzymes responsible for the deinococci's radioresistance. Does the resistance mechanism rely upon a set of unique or highly efficient DNA repair enzymes, or does it rely upon a unique mechanism of regulating and/or organizing the action of DNA repair enzymes? Although this dissertation does not definitively answer this question, it does provide additional evidence supporting
the hypothesis that regulation of common DNA repair enzymes in *D. radiodurans* is a critical component of the mechanism by which *D. radiodurans* survives radiation damage.

I. Summary Description of *Deinococcus radiodurans*

1. General Characteristics

The genus *Deinococcus* consists of four characterized species—*D. radiodurans, D. proteolyticus, D. radiopugnans, and D. radiophilus*. *D. radiodurans* is the type species for the genus (8, 50). All members of the genus are non-sporulating and nonmotile. In liquid culture they form pairs or tetrads. Growth on solid media produces convex, smooth colonies which vary from pink to red. *D. radiodurans* stains gram positive, but has a complex cell envelope similar to that of gram negative organisms. Cells are chemoorganotrophic with respiratory metabolism, and they are typically grown with aeration in TGY broth (0.5 percent tryptone, 0.1 percent glucose, 0.3 percent yeast extract). The optimal growth temperature is 30°C. Growth slows near 37°C and ceases at 45°C.

The *D. radiodurans* chromosome is estimated to be 3 x 10⁸ base pairs (22). The organism is multi-genomic (28). In exponentially growing cells, as many as ten complete copies may exist per cell. A minimum of four identical copies is present per stationary phase cell. The
chromosome is a single covalently-closed circular molecule. The base composition of all deinococcal species is characterized by a GC content of 65 to 71 percent. Stable naturally-occurring plasmids have been found in all species examined (54). Most plasmids are present in low copy number and are larger than 20 kb. These plasmids have not been extensively studied, and their function in cellular metabolism is unknown. Stable shuttle vectors capable of replicating in D. radiodurans and E. coli have been constructed by inserting an E. coli plasmid into one of several D. radiodurans plasmids and removing sequences not needed for plasmid replication (54).

2. Taxonomy and Phylogeny

The deinococci morphologically resemble members of the genus Micrococcus, and were originally classified as such. However, taxonomic and chemotaxonomic studies of the Deinococcaceae suggested that this classification was incorrect. Subsequent phylogenetic analysis of deinococcal 16S and 5S rRNA sequences confirm that the Deinococcaceae are not related to the Micrococcaceae (30, 62, 65). The genus Deinococcus is specifically related to the gram negative genera Deinobacter and Thermus. Deinobacter strains are radiation resistant rod-shaped organisms that exhibit chemotaxonomic characteristics very similar to those of Deinococcus. The genera Deinococcus
and *Deinobacter* make up the family *Deinococcaceae*. No phenotypic or chemotaxonomic relationships are obvious between *Deinococcus* and the genus *Thermus*. The three genera form one of the ten phyla that are the *Bacteria*.

3. Habitat

The natural habitat of the deinococci has not been defined. Although *Deinococcus* strains have been identified in a variety of locations world-wide, ecological studies have been limited and uninformative (35, 40). The deinococci require a rich organic environment. Deinococci have been isolated from soil (35, 50, 52), animal feces (31), processed meats (21, 35), and sewage (31). It is, however, possible that in nature the deinococci grow in the presence of other microorganisms that provide them with complex organic nutrients. This would, accordingly, expand the possible environments that the deinococci could exploit, explaining their isolation from unusual habitats. *D. radiopugnans*, for example, has been identified as part of an endolithic community in the Ross desert of Antarctica. This location is considered to be the most hostile terrestrial habitat (51), as bacterial communities living there are subject to dehydration, severe cold, and intense UV light, receiving between 425 and 1000 hours of sunlight annually. Barring nutritional requirements, *D. radiodurans* as a vegetative organism is
well suited for this environment. It is highly resistant to desiccation, exhibiting ten percent viability after storage in a desiccator for six years (49). It is also highly UV resistant (49).

4. Natural Transformation

The study of *D. radiodurans* has been hampered by a lack of genetic tools appropriate for use with this organism, as many are not applicable to the study of *D. radiodurans*. *D. radiodurans* displays no evidence of conjugation (46). Phage capable of infecting *D. radiodurans* have never been identified, despite a great deal of effort (46, 56). Fortunately, *D. radiodurans* is relatively easy to manipulate using natural transformation. *D. radiodurans* is the only member of the deinococci that is naturally transformable (59). As a result, *D. radiodurans* is the more tractable species for the study of extreme radioresistance in the Deinococcaceae.

In *D. radiodurans* transforming DNA is readily taken up and incorporated into the chromosome with marker-specific efficiencies from 0.01-3.0 percent when cells are transformed in liquid culture in the presence of calcium (59). The high transformation efficiency exhibited by *D. radiodurans* greatly facilitates the isolation of DNA fragments carrying genes involved in DNA damage.
A genomic library generated from the wild type strain can be efficiently screened for clones capable of restoring the wild type phenotype to mutant strains. As part of the transformation process, the mutant allele is replaced by the wild type sequence in a recombinational event. Moseley and coworkers (2) were the first to use this technique when they cloned the wild type gene necessary for *D. radiodurans*' mitomycin resistance. They transformed individual cosmids into a mitomycin sensitive strain, *D. radiodurans* 302. Transformants were then plated onto media containing mitomycin C. Surviving colonies identified the cosmid carrying the wild type sequence that corresponded to the allele responsible for mitomycin C sensitivity. Once the cosmids which carried mitomycin resistance genes were identified, the gene was subcloned by transforming restriction fragments obtained from that cosmid into strain 302. Moseley's group found that the efficiency of transformation remained high until fragment size dropped below 1.2 kb (2).

Additionally, natural transformation has been used to evaluate the recombination proficiency of *D. radiodurans* strains (24, 45) and to determine allelism among *D. radiodurans* mutants with similar phenotypes (19). Since natural transformation requires homologous recombination, it has generally been assumed that transformable strains
of *D. radiodurans* are recombination proficient. Recombination proficiency is determined by attempting to transform the strain being tested with an antibiotic resistance marker. Successful transformation will then indicate that the strain is recombination-proficient. To evaluate allelism, chromosomal DNA from a strain exhibiting a mutant phenotype is used to attempt to transform another strain exhibiting a similar mutant phenotype. For this evaluation "dot" transformation can be used. Dot transformation is possible in strains of *D. radiodurans* because they remain fully competent throughout exponential growth (59). The protocol for dot transformation is described in detail in Chapter Two. Briefly, exponential phase *D. radiodurans* cells are plated onto a rich medium, dotted with transforming DNA, and allowed to grow into a lawn. The lawn is then replica plated and selective pressure is applied. Successful transformants appear within the area where the transforming DNA was dotted. Dot transformation allows investigators to avoid the tedium associated with conducting the large number of transformations needed when screening a genomic library. Dot transformation also allows investigators to qualitatively evaluate alleleism among mutant strains by direct visualization of a positive or negative area of transformation. That is, successful
transformation to wild type appears as a dot of heavy growth on the plate. Positive transformation provides evidence that the two mutant strains have different genotypes, that is, the phenotype they display is the consequence of different mutations. The failure of chromosomal DNA from one mutant strain to restore a wild type phenotype to the other mutant strain indicates one of two alternative possibilities: i) that the mutations found in the donor and recipient strains affect the same site, or ii) that these mutations affect different sites closely linked to each other. The protocols used to assess alleleism in D. radiodurans ionizing radiation sensitive (IRS) mutants are also discussed in detail in Chapter Two of this dissertation.

5. DNA Damage Tolerance

D. radiodurans tolerates a variety of DNA damaging agents, with unusually high resistance to ionizing radiation and UV light (260 - 280 nm) (41, 43). A comprehensive survey of D. radiodurans' response to different DNA damaging agents has not been reported. However, D. radiodurans is known to exhibit resistance to killing by cross-linking agents, nitrous acid, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), and 4-nitroquinoline-N-oxide (57, 58). In contrast, D. radiodurans cultures are as sensitive as E.
coli to some simple alkylating agents such as N-methyl-N-nitrourethane, ethyl methane sulphonate, and \(\beta\)-propriolactone. There is also a single report that \(D.\ radiodurans\) is unusually sensitive to near UV light (300 - 400 nm)\(^9\). Treatment of \(D.\ radiodurans\) cultures with most DNA damaging agents, even at highly lethal doses, fails to increase the frequency of mutation above that of the spontaneous mutation rate. Alkylating agents appear to be the only DNA damaging agents able to effectively induce mutagenesis in \(D.\ radiodurans\).

A. Ionizing Radiation

The deinococci's most distinguishing characteristic is their ability to tolerate ionizing radiation. This characteristic allowed their initial isolation and is still used to isolate deinococcal species from the environment \(^{31}\). An ionizing radiation survival curve for wild type \(D.\ radiodurans\) strain R1 is characterized by a shoulder of complete viability to 5,000 Gy \(^{47}\). At doses above 5,000 Gy, an exponential loss of viability is observed. Exponential phase cultures survive 15 kGy ionizing radiation \(^{49}\). There are reports of other deinococcal strains surviving as much as 50 kGy \(^5\). The \(D_1\) dose following exposure to ionizing radiation for \(D.\ radiodurans\) R1 in exponential growth is approximately 6,000 Gy. Smith et al. \(^{56}\) have estimated that this dose
of ionizing radiation induces approximately 200 DNA double strand breaks (dsbs), over 3,000 single strand breaks, and greater than 1,000 sites of base damage per \textit{D. radiodurans} genome. The effect of 5,000 Gy ionizing radiation on the \textit{D. radiodurans} chromosome is dramatically illustrated by comparing chromosomal DNA preparations from irradiated and unirradiated cultures using pulsed-field gel electrophoresis (PFGE) (22). In irradiated cultures, the chromosome is reduced from a single $3 \times 10^8$ bp fragment to a wide band of fragments 50 kb or smaller in size. Viability for the irradiated cells containing the fragmented DNA remains at 100 percent.

\subsection*{B. UV Light}

Wild type \textit{D. radiodurans} cultures are extremely resistant to UV light (260 nm) and will survive doses as high as 1,000 J/m$^2$ (47). UV survival curves are characterized by a shoulder of resistance extending to approximately 500 J/m$^2$. Cultures irradiated with doses lower than 500 J/m$^2$ do not exhibit any significant loss of viability. Beyond 500 J/m$^2$, there is an exponential reduction in survival. The D$_{0}$ dose is the dose that, on average, is required to inactivate a single colony forming unit (CFU). The D$_{0}$ for \textit{D. radiodurans} is reported to be between 550 and 600 J/m$^2$ for exponentially-growing cultures. Approximately one percent of the irradiated
cell's thymine is present as part of a pyrimidine dimer after exposure to 500 J/m UV (6, 63). Assuming that the D. radiodurans genome is 3 x 10^9 base pairs with 67 percent GC content, this one percent would represent as many as 5,000 thymine-containing pyrimidine dimers per genome.

C. Cross-linking Agents

D. radiodurans is resistant to the cross-linking agent mitomycin C and will survive incubation with high concentrations of this antibiotic. In the presence of 20 \( \mu \)g/ml mitomycin for ten minutes at 30°C, there is no loss of viability. After forty minutes of incubation at this concentration one percent of the culture remains viable (32). Although the number of cross-links formed are difficult to estimate under these conditions, Kitayama (32) has reported that greater than 90 percent of the chromosomal DNA isolated from cultures treated for ten minutes exists as fragments of non-denaturable double-stranded DNA with an average molecular weight of 2 x 10^9 Daltons (Da), indicating that at least 100 mitomycin C-induced crosslinks form per genome at this dose.

II. The Enzymology of DNA Damage Repair

The enzymology of deinococcal DNA repair has not been defined completely. A handful of studies in the last forty years have attempted to detail the biochemistry of
processes associated with DNA repair. Thus far, only three types of DNA repair have been described: i) nucleotide excision repair, ii) base excision repair, and iii) recombinational repair. Photoreactivation and error-prone repair systems as observed in \textit{E. coli} have not been identified in deinococcal strains (46). There are no reports which identify systems for mismatch correction in the deinococci or identify an adaptive response in these organisms toward alkylation damage.

1. Evidence for Nucleotide Excision Repair

Studies of \textit{D. radiodurans} R1 have shown that resistance to UV-induced DNA damage is partially mediated by the activity of a protein, endonuclease alpha (46). Endonuclease alpha, the product of the \textit{mtcA} and \textit{mtcB} genes, recognizes a broad range of DNA damage, incising DNA at or near the site of damage (46). Agostini and Minton (1) have cloned the wild type sequences corresponding to \textit{mtcA} and \textit{mtcB}. \textit{mtcA} and \textit{mtcB} are now known to be mutations in the same coding sequence. \textit{mtcA} and \textit{mtcB} share sixty percent homology with the coding sequences of the \textit{uvrA} genes of \textit{E. coli} and \textit{Micrococcus luteus}. The \textit{mtcA} and \textit{mtcB} alleles are now designated \textit{uvrA1} and \textit{uvrA2}, respectively. The homology suggests that endonuclease alpha functions in a way similar to the UvrABC exinuclease of \textit{E. coli}. There is no evidence that
D. radiodurans expresses homologues of the uvrB and uvrC gene products, but an effort to search for these proteins or their coding sequences has not been described.

2. Evidence for Base Excision Repair

Mechanisms of DNA repair involving base excision in D. radiodurans have not been examined in detail, even though this type of repair function would be expected to exist in an organism capable of withstanding ionizing radiation-induced DNA damage. Reports describe an apurinic/apyrimidinic (AP) endonuclease activity (39), a uracil-N-glycosylase activity (39), a DNA deoxyribophosphodiesterase (dRPase) activity (48), and a thymine glycol glycosylase activity (48) in cell extracts of D. radiodurans. The AP endonuclease has been partially purified and has a molecular weight of 34.5 kDa. The purified fraction does not exhibit glycosylase activity toward DNA containing uracil, alkylated bases, or UV photoproducts and does not require a metal, as it was active in the presence of EDTA. The uracil-N-glycosylase is specific for uracil-containing DNA and has no metal requirement. The molecular weights of the dRPase and the thymine glycol glycosylase are estimated at between 25 and 30 kDa, respectively. The dRPase requires Mg\(^{2+}\) for activity. The thymine glycol glycosylase does not require a metal for activity.
Additional reports by Gutman et al. (27) suggest that *D. radiodurans* expresses a pyrimidine-dimer DNA glycosylase (PD DNA glycosylase) analogous to the PD DNA glycosylases isolated from *Micrococcus luteus* or bacteriophage T4. They have shown that expression of the *denV* gene of T4 in *uvrA uvs* and *uvs* strains of *D. radiodurans* can partially restore UV resistance to these strains. This indicates that the *uvs* gene product, also called endonuclease beta, has a function similar to the *denV* gene product. Endonuclease S is a 36 kDa monomeric protein that has been partially purified (18). Its activity does not require ATP. It also exhibits a novel requirement for Mn\(^{2+}\) ions. Three mutations in loci designated *uvsC*, *uvsD*, and *uvsE* have been isolated that inactivate endonuclease beta activity (19). It has been suggested that each mutation affects a separate coding sequence, because genomic DNA from each *uvs* mutant will restore wild type UV resistance to the other two *uvs* mutants. It is, however, a possibility that *uvsC*, *uvsD*, and *uvsE* are alleles within the same gene just as *mtcA* and *mtcB* were shown to be (1).

Endonuclease beta is similar to characterized PD DNA glycosylases, because it catalyzes an incision adjacent to pyrimidine dimers and facilitates their removal (17). However, the action of this enzyme *in vitro* is not
associated with an AP lyase activity (17). The *M. luteus* and T4 PD DNA glycosylase activities result in the cleavage of the N-glycosyl bond of the 5' base in the pyrimidine dimer, producing a structure that will release a free thymine upon photoreversal. Free thymine is not released when photoreversal is attempted on UV-irradiated DNA treated with endonuclease beta. Therefore, the action of endonuclease beta does not appear to cleave this bond.

3. Evidence for Dsb Repair

When an exponential phase culture of *D. radiodurans* is exposed to 5,000 Gy ionizing radiation, greater than 150 DNA dsbs are introduced into the chromosome (56). The dsbs are repaired without loss of viability and the chromosome is apparently reassembled without compromising the linear continuity of the genome. *D. radiodurans rec* strains are ionizing radiation sensitive and incapable of repairing DNA dsbs (10). They are also sensitive to mitomycin C and UV light. This indicates that the rec gene product is involved in repair pathways common to other types of DNA damage. The rec gene has been cloned and sequenced. The product shares 56 percent identity with *E. coli*’s RecA (24). Despite the similarity between the two proteins, the recA from *D. radiodurans* will not complement an *E. coli* recA mutant. It has been reported that the expression of the deinococcal RecA protein is
toxic to *E. coli* and that even low level expression is lethal suggesting a fundamental difference in the activity of the two proteins. Daly and Minton have followed the kinetics of DNA dsb repair in stationary phase cells exposed to 17.5 kGy using PFGE (10). They estimate the cells suffer approximately 100 DNA dsbs at this dose. In the first 1.5 hours post-irradiation, they report a recA-independent increase in the molecular weight of chromosomal DNA of the irradiated population. They report a recA-dependent increase in molecular weight three hours later, continuing for the next twenty-nine hours until the chromosome is restored to its normal size. Daly and Minton infer that the recA-independent reassembly of the chromosome is the consequence of a single strand annealing reaction such as that catalyzed by the RecE and RecT proteins of *E. coli*, but they present no evidence to support this prediction. The reassembly of the chromosome post-irradiation proceeds in a stepwise fashion, as chromosomal fragments become larger over time.

4. Evidence for a Deinococcal DNA Polymerase I Homologue

Gutman et al. (26) have cloned and sequenced the gene which encodes the DNA polymerase necessary for DNA damage resistance in *D. radiodurans*. The *D. radiodurans*' *pol* gene product shares 51.1 percent homology with DNA polymerase I of *E. coli*. Insertional mutagenesis of the
pol coding sequence generates a strain that is extremely sensitive to UV, ionizing radiation and mitomycin C (26). pol strains of D. radiodurans can be restored to DNA damage resistance by the intracellular expression of a clone of E. coli's DNA polymerase I (25). Therefore, the pol gene product of D. radiodurans and E. coli's DNA polymerase I are assumed to have the same activities.

III. DNA Damage Sensitive Strains of D. Radiodurans

Although D. radiodurans strains are extremely resistant to many DNA damaging agents, it is still possible to induce mutation in cultures treated with simple alkylationing agents such as MNNG. Most of the known base substitution mutations that render D. radiodurans sensitive to DNA damage have been generated using alkylationing agents.

To isolate mutants, individual colonies from mutagenized cultures of D. radiodurans must be screened for strains exhibiting sensitivity to a DNA damaging agent. Mutagenesis by this means, though labor intensive, remains the only effective method for randomly generating D. radiodurans mutants. Transposon mutagenesis has not been reported in D. radiodurans, because the drug resistance markers used to identify successful transposition are probably not expressed. It is presumed that these drug markers are not expressed because the
promoter structure of *D. radiodurans* is different from that of other prokaryotes and is, therefore, not recognized by the RNA polymerases of other species (55). The *D. radiodurans* RNA polymerase does not recognize the promoters of other species (55).

1. UV and Mitomycin C Sensitive Strains

Screens for mitomycin C and UV sensitive strains of *D. radiodurans* have been reported (42, 46). The first strains sensitive solely to mitomycin C were derived from *D. radiodurans* RL. The loci inactivated in these strains were identified as *mtcA* and *mtcB* (44), and are now known as *uvrA1* and *uvrA2*, respectively. Additional mitomycin C sensitive mutants generated by Kitayama and colleagues indicated that there were as many as five more independent loci, designated *mtcC* through *mtcG*, associated with the repair of mitomycin C-induced DNA damage (33). The *mtcC* through *mtcG* mutants have not been characterized.

The *uvrA* strains of *D. radiodurans* are not sensitive to UV light or ionizing radiation. UV sensitivity requires the inactivation of a second locus, designated *uvs*, before a *uvrA* strain becomes UV sensitive (19, 46). Three *uvs* loci, *uvsC*, *uvsD*, and *uvsE*, have been identified. *uvrA uvs* double mutants are sensitive to UV light. Strains carrying only a *uvrA* or a *uvs* mutation exhibit near wild type levels of UV resistance, but are
each sensitive to mitomycin C. The UV resistance exhibited by a uvrA or uvs mutant indicates that the uvrA and uvs gene products have overlapping activities and encode functionally redundant proteins with respect to UV resistance. uvs mutants exhibit wild type resistance to mitomycin C and ionizing radiation. uvrA uvs double mutants are sensitive to mitomycin C and UV radiation, but are as resistant to ionizing radiation as wild type D. radiodurans.

2. IRS Strains

Forty-five thousand MNNG-treated colonies of the uvrA strain D. radiodurans 302 were screened for ionizing radiation sensitivity. Subsequently, forty-nine putative IRS strains were identified (60). The IRS strains are the largest collection of radiosensitive mutants ever assembled in D. radiodurans. As such, they represent a previously unavailable resource for the study of this organism.

Two new loci, irrB and irrI, have been identified within the IRS mutant collection. Inactivation of either locus results in a partial loss of resistance to ionizing radiation (60). The magnitude of this loss is locus specific and differentially affected by inactivation of the uvrA gene product. The irrB and irrI mutations also reduce D. radiodurans resistance to ultraviolet radiation,
this effect being most pronounced in an \textit{irrI uvrA}'
background.

The functions of the \textit{irrI} and \textit{irrB} gene products are
unknown, but there is preliminary evidence that the \textit{irrI}
gene product may be a regulatory protein. This is further
discussed in Chapter Four. A \textit{uvrA irrI} strain is only
slightly more sensitive to UV than strain 302, its \textit{irrI}'
parent. In contrast, an \textit{irrI} strain that is \textit{uvrA}' is
extremely sensitive to UV light, exhibiting a dramatic
reduction in survival following UV doses that are
sublethal to \textit{uvrA irrI} and \textit{uvrA} strains. This result
suggests that the \textit{irrI} gene product regulates either the
activity of endonuclease alpha or an enzymatic activity
that arises subsequent to the action of endonuclease
alpha.
CHAPTER TWO - GENETIC CHARACTERIZATION OF FORTY IRS STRAINS OF DEINOCOCCUS RADIODURANS: LINKAGE INFORMATION FROM TRANSFORMATION

I. Introduction

During previous studies of *D. radiodurans*, forty-nine putative IRS strains were isolated with the intention of using these strains to define the enzymatic components involved in deinococcal ionizing radiation resistance. This collection of strains is further characterized here. The mutations responsible for the IRS phenotype fall into sixteen different linkage groups. All but three of the IRS strains are recombination proficient. Three of the IRS strains carry mutations in the *D. radiodurans* pol locus. Five of the strains in this collection recover from ionizing radiation-induced DNA damage at a significantly slower rate than that of their parent, *D. radiodurans* 302.

II. Materials and Methods

1. Bacterial Strains, Plasmids, and Growth Media

Bacterial strains and plasmids used in this study are listed in Table 3 of the Appendix. Plasmids were routinely propagated in *E. coli* DH5α-MCR. Plasmids were isolated by alkaline extraction. All *D. radiodurans* strains were grown at 30°C in TGY broth (0.5 percent...
tryptone, 0.3 percent yeast extract, 0.1 percent glucose) or on TGY agar (TGY agar containing 1.5 percent agar).

2. Mutagenesis and Isolation of IRS Strains.

Exponentially growing cultures of *D. radiodurans* 302, a uvrA strain, were treated with 20 μg of MNNG per ml and incubated for 2 hours at 30°C with shaking (60). A 100 μl aliquot of mutagenized cells was diluted in 25 ml of TGY and incubated for an additional eighteen hours. The resulting culture was diluted and plated at 200 to 300 CFU per plate. The mutagenized population was screened for ionizing radiation sensitivity by patching individual colonies onto TGY plates and exposing the plates to 5,000 Gy of gamma radiation (Co source, 14.4 Gy/min, 22°C). Colonies of untreated *D. radiodurans* 302 patched onto each plate served as a control. IRS mutants were identified by comparing the growth of mutagenized colonies with that of strain 302 48 hours after irradiation. Putative mutants were streaked to isolation, and the IRS phenotype was confirmed by irradiating cultures grown from isolated colonies.

3. Quantification of Ionizing Radiation Resistance

*D. radiodurans* cultures in the exponential growth phase were evaluated for their ability to survive ionizing radiation. Cultures were divided into 1 ml aliquots, placed in microcentrifuge tubes, and exposed to a Co
source. Cultures were removed from the source after an accumulated dose of 5,200 Gy. Irradiated cells were diluted, plated in triplicate on TGY agar plates, and incubated for five days at 30 C before being scored for survivors. The size of the population irradiated was determined by measuring the titer of each culture immediately prior to irradiation. Percent survival for individual IRS strains was calculated by dividing the number of survivors (expressed as CFU per milliliter) by that strain's titer prior to irradiation, and then multiplying the resulting fraction by 100.

4. Chromosomal DNA Isolation

TGY broth (500 ml) was inoculated with a 2 ml overnight culture (approximately \(2 \times 10^7\) CFU/ml) of \(D.\) \(radiodurans\). After 18 h, the 500 ml cultures were harvested by centrifugation at 4 C at 3000 \(x\) \(g\) for 15 min. Pellets were suspended in 25 ml absolute ethanol to remove \(D.\) \(radiodurans'\) outer membrane. The ethanol-stripped cells were collected by centrifugation at 4 C at 3000 \(x\) \(g\) for 15 min and the pellet suspended in 1 ml TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). Two milligrams of lysozyme was added to the stripped cells. This mixture was incubated at 37 C for 30 min. Five milliliters of a pronase E solution (two percent sodium dodecyl sulfate, 0.1 M EDTA pH 8.0, and 4 mg Pronase E) was added to the
lysozyme-treated cells and incubated for 3 h at 50°C. Lysed cells were transferred to a centrifuge tube and extracted twice with an equal volume of phenol:chloroform (1:1). The DNA was precipitated by adding 1 ml of 3 M sodium acetate (pH 7.0) and 20 ml of ice-cold absolute ethanol to the extracted material. The DNA was spooled out using a curved glass rod and washed twice with seventy percent ethanol. The DNA was air dried and dissolved in 4 ml of TE buffer (pH 8.0), and then stored at 4°C. This procedure yielded approximately 400 mg chromosomal DNA per 500 ml culture. When analyzed by PFGE this preparation routinely generated 40 to 50 kb DNA fragments.

5. Dot Transformation

The dot transformation protocol used in this study is summarized in Fig. 2.1. A 100 μl aliquot of the D. radiodurans culture in the exponential growth phase was spread onto a TGY plate and incubated for four to six hours at 30°C. Transformations were performed by dotting three to seven μg of either chromosomal or plasmid DNA onto the plate. Twenty-four hours later, the bacterial lawn was replica plated onto TGY agar and selective pressure was applied. Plates were exposed to 7,500 Gy of gamma radiation to select for ionizing radiation resistance. Plates were examined each day for the next
DOT TRANSFORMATION

Inoculate 2 ml TGY broth with *D. radiodurans* strain.

Incubate with vigorous shaking for 18h at 30°C.

Pellet culture and resuspend in TGY broth. Spread culture onto TGY agar plate. Incubate for 1h at 30°C.

Dot plate surface with at least 200ng of transforming DNA. Incubate for 1 day at 30°C.

Apply selection.

Fig. 2.1. Diagrammatic representation of the dot-transformation procedure.
five days and scored by noting colony formation within the area where the DNA had been dotted.

6. Growth Measurement

A 500 µl aliquot of an overnight culture of each strain was transferred to 50 ml of TGY broth in a 250 ml flask. The culture was grown at 30°C with vigorous shaking (200 rpm). At two (t.) and four (t.) hours post-inoculation, an aliquot of the growing culture was removed and serially diluted in 10 mM MgSO₄. The dilutions were spread onto TGY agar. Spread plates were incubated at 30°C for five days, and the number of CFU was determined. The doubling time, $g$, was calculated from the equation $g = \ln 2/k$. The growth rate constant, $k$, was calculated from the equation $k = (\log_{10} N_t - \log_{10} N_i) 2.303/\Delta t$, where $N_i = \text{CFU/ml at } t_i$ and $N_t = \text{CFU/ml at } t_t$.

III. Results

1. IRS Mutants of *D. radiodurans* 302

Isolation of IRS strains was described in an earlier report (60). To confirm the IRS phenotype, each IRS strain's ability to survive exposure to 5,200 Gy of gamma radiation was compared with that of the parent strain. Exponentially growing cultures of each putative IRS strain were irradiated, serially diluted, plated in triplicate on TGY agar, and scored for survivors five days after plating. Fig. 2.2 depicts the results of this assessment.
Fig. 2.2. Percent survival of IRS mutants following exposure to 5,200 Gy gamma radiation.
Values are the mean percent survivals ± standard deviations of six replicates from two experiments. Strain 302 displayed approximately eighty-four percent survival. Forty-three of the forty-nine putative IRS strains exhibited reduced survivals relative to 302 in this assay. IRS46 was the most ionizing radiation resistant of the IRS strains with sixty-five percent survival. Twenty-nine of the IRS strains demonstrated survivals of ten percent or less. IRS26, IRS27, IRS33, IRS34, IRS38 and IRS41 were the most sensitive strains with less than 0.1 percent survival at the dose administered. The survival of *D. radiodurans* R1, the wild type strain, did not differ significantly from that of strain 302.

Six of the strains that were originally designated IRS failed to exhibit enhanced sensitivity to gamma radiation in this assay and are not included in Fig. 2.2. The IRS phenotype for the strain originally designated IRS3 could not be confirmed, and this strain was not investigated further. The other five strains displayed a novel phenotype that explained their misclassification as IRS strains.

2. Mutants of *D. Radiodurans* That Recover from DNA Damage More Slowly Than Strain 302

Five of the original forty-nine IRS strains evaluated in this study displayed an unusual phenotype. They
recovered from gamma radiation more slowly than their parent strain, 302. When the survival of these strains, originally designated IRS1, IRS2, IRS5, IRS16, and IRS17, was determined two days post-irradiation, the strains appeared to be highly susceptible to gamma radiation (Fig. 2.3), with none of the five strains exhibiting more than five percent survival. However, when the plates used to quantify survival were returned to the incubator and recounted five days post-irradiation, the strains did not exhibit enhanced ionizing radiation sensitivity (Fig. 2.3). Surviving cells could not form detectable colonies within two days of irradiation, but visible colonies did form within five days. The low level of survival calculated after two days was therefore an artifact of the slow appearance of survivors. These five strains have been reclassified and are now referred to as slow-recovery or SLR strains. The new strain designations are listed in Table 3 in the Appendix.

The slow recovery phenotype was not observed when evaluating the survival of R1, 302 or the majority of the IRS strains following exposure to gamma radiation. Three IRS strains did, however, exhibit slow recovery. There was an approximately thirty-fold increase in the number of survivors between two and five days post-irradiation in cultures of IRS6, IRS35, and IRS37.
Fig. 2.3. Survival of SLR strains following exposure to 5,200 Gy gamma radiation. Solid bars represent percent survival based on the number of detectable survivors 48 hours post-irradiation. Hatched bars represent percent survival based on the number of detectable survivors 120 hours post-irradiation. Each value is the mean ± the standard deviation of twelve replicates from three experiments.
Two possibilities seemed likely to account for the slow recovery phenotype: i) the SLR strains acquired mutations that resulted in slow growth, or ii) the SLR strains acquired mutations that slowed some aspect of the repair process needed to recover from ionizing radiation-induced DNA damage. The first possibility was evaluated by determining the generation time of the SLR strains and comparing that value with the generation time of strains R1 and 302. Those results are presented in Table 1. The growth rate of SLR2, SLR4, and SLR5 did not differ from that of 302. In contrast, SLR1 and SLR3 grew from 1.5 to 2 times more slowly than 302.

3. Forty of the IRS Strains Are Transformable

Exponentially growing cultures of *D. radiodurans* readily take up and incorporate homologous DNA into their chromosome (32), and functional deinococcal RecA is required for this process (32, 41). The IRS strains were screened for loss of RecA activity by determining if they could be transformed. Transformation can occur by simply dotting as little as 200 ng of transforming DNA onto a lawn of exponentially growing *D. radiodurans* cells (Fig. 2.4 A). This transformation procedure was termed "dot" transformation. The dot transformation procedure is depicted in Fig. 2.1. Transforming DNA was isolated from *D. radiodurans* LS18, a streptomycin resistant strain.
### Table 1. Generation times of *D. radiodurans* SLR strains

<table>
<thead>
<tr>
<th></th>
<th>R1</th>
<th>302</th>
<th>SLR1</th>
<th>SLR2</th>
<th>SLR3</th>
<th>SLR4</th>
<th>SLR5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean generation time (h)± SD of strain*</td>
<td>1.0±0.03</td>
<td>1.5±0.3</td>
<td>2.2±0.3°</td>
<td>1.5±0.2</td>
<td>2.9±0.7°</td>
<td>1.5±0.04</td>
<td>1.2±0.02</td>
</tr>
</tbody>
</table>

* See appendix for descriptions of the strains used in this analysis. Each value is the mean ± the standard deviation of four experiments.

° With Student's t test, the mean generation times calculated for strains SLR1 and SLR3 were found to differ significantly from that calculated for strain 302 at a 95% level of confidence.
Fig. 2.4. Pattern of growth on *D. radiodurans* spread plates after dot transformation. A) *D. radiodurans* R1 dot transformed with 200 ng of chromosomal DNA from LS18, a streptomycin-resistant isolate of *D. radiodurans* R1. Transformants were selected on TGY agar containing 50 μg of streptomycin per ml. B) Pattern of growth on a IRS18 plate dot transformed with 500 ng of chromosomal DNA from strains IRS41 to IRS47. Following replica plating, the IRS18 lawn was exposed to 6,000 Gy gamma radiation. Areas of growth indicate successful transformation to ionizing radiation resistance.
previously isolated. The streptomycin resistance marker allows resistance to 100 μg/ml of streptomycin in normally sensitive D. radiodurans cells. An attempt was made to transform each IRS strain to streptomycin resistance using chromosomal DNA isolated from LS18. All but three of the IRS strains were successfully transformed to streptomycin resistance with an efficiency of approximately 0.1 percent. It was therefore assumed that all transformable strains expressed a functional RecA protein and were proficient in homologous recombination.

IRS26, IRS27, and IRS47 could not be transformed by this protocol. IRS26 and IRS27 were found to be quite sensitive to gamma radiation. They were 100- and 300-fold more sensitive than 302, respectively, to a 5,200-Gy dose (Fig. 2.2). In contrast, IRS47 was only four-fold more sensitive than 302.

4. Identification of Sixteen Linkage Groups among the Recombination Proficient IRS Strains

Linkage between mutations represented within the recombination proficient IRS strains was determined using transformation. An aliquot from an exponential-phase culture of each IRS strain was spread onto a TGY agar plate, and 5 μg of genomic DNA from eight to twelve other IRS strains was dotted in separate locations on the plate. After a forty-eight hour incubation at 30°C, the lawn that
formed was replica plated onto fresh TGY plates and gamma irradiated at 7,500 Gy to select for cells transformed to wild type levels of ionizing radiation resistance. The resulting growth pattern for the selection of wild type transformants is shown in Fig. 2.4 B. Restoration of ionizing radiation resistance in the recipient strain indicates allelic substitution. The mutation responsible for the recipient strain's ionizing radiation sensitivity is replaced with the wild-type sequence provided by the donor DNA in a recombinational event. Chromosomal DNA from each IRS strain was tested for the ability to restore a radioresistant phenotype to all other IRS strains. The results of those analyses are summarized in Table 2. Those strains that failed to restore radioresistance to each other were grouped together. Each group was assigned a letter designation. For example, the five strains in group K cannot restore radioresistance to each other, but do restore resistance to all other recombination-proficient IRS strains. By this analysis, the IRS strains were divided into sixteen different linkage groups.

5. The Ordering of Mutations within the pol Linkage Group

The physical distance separating the mutations that define each linkage group appears to be small. This fact is most evident when linkage groups C and D were examined.
Table 2. Linkage groups identified among IRS strains by dot transformation

| Group A: | IRS9 | Group L: | IRS8  |
| Group B: | IRS18 | IRS43  |
| Group C: | IRS7  | IRS44  |
| Group D: | IRS33 | Group M: | IRS14 |
| Group E: | IRS38 | IRS15  |
| Group F: | IRS24 | IRS21  |
| Group G: | IRS34 | IRS29  |
| Group H: | IRS32 | IRS48  |
| Group I: | IRS25 | Group N: | IRS4  |
| Group J: | IRS42 | IRS14  |
| Group K: | IRS49 | IRS30  |
| Group L: | IRS10 | IRS40  |
| Group M: | IRS12 | Group O: | IRS12 |
| Group N: | IRS25 | IRS39  |
| Group O: | IRS39 | IRS39  |
| Group P: | IRS46 | IRS45  |

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A previous set of plasmid transformation experiments demonstrated that IRS7, IRS33, and IRS38 carry different mutations within the *pol* gene (60). The *pol* mutations carried by IRS7, IRS33, and IRS38 were arbitrarily designated the *pol-1*, *pol-2*, and *pol-3* mutants, respectively. Linkage group data from this experiment demonstrates that IRS33 and IRS38 form a linkage group that does not include IRS7. This suggests that *pol-2* and *pol-3* mutations found in IRS33 and IRS38, respectively, are closer to each other than they are to the *pol-1* mutation found in IRS7. Genomic DNA isolated from IRS7 transforms IRS38 to wild type levels of ionizing radiation resistance with an efficiency of approximately 0.1 percent. This efficiency is identical to that obtained when chromosomal DNA isolated from wild type *D. radiodurans* R1 was used to transform IRS38 to ionizing radiation resistance. In contrast, DNA isolated from IRS33 did not transform IRS38 to ionizing radiation resistance at a detectable frequency. Presumably, the sites of the *pol-2* and *pol-3* mutations are so close that replacement of the *pol-3* mutation with wild type sequence derived from IRS33 results in the simultaneous incorporation of the *pol-2* mutation. The transformant remains IRS. The relative positions of the *pol-1*, *pol-2*,

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and pol-3 mutations are represented diagrammatically in Fig. 2.5.

IV. Discussion and Conclusions

The isolation of forty-nine putative IRS derivatives of *D. radiodurans* strain 302 was previously reported (60). In this study, the isolates were further characterized. Evaluation of the forty-nine strains revealed that forty-three were truly sensitive to ionizing radiation when compared with their parent strain (Fig. 2.2) and that the other six strains had been misclassified as IRS in our initial study.

Five of the six misclassified strains exhibited a mutant phenotype that gave the appearance of ionizing radiation sensitivity. These strains, which were originally designated IRS1, IRS2, IRS5, IRS16, and IRS17, recover from gamma irradiation more slowly than does the parent strain. For this reason, we have chosen to rename these strains SLR1 through SLR5 (Table 3 in the Appendix). Two of the strains, SLR1 and SLR3, grow approximately twice as slowly as strain 302, and this may account for the slow appearance of colonies post-irradiation. This explanation suggests that the mutations found in SLR1 and SLR3 affect growth without affecting the cell's ability to recover from radiation-induced DNA damage. In contrast, SLR2, SLR4, and SLR5 exhibit normal growth rates.
Fig. 2.5. Representation of the relative positions of the pol-1, pol-2, and pol-3 alleles within the *D. radiodurans* pol coding sequence. The numbers 1, 2, and 3 indicate the positions of the respective mutations. Those portions of the pol gene associated with plasmids pPG9, pPG11, and pPG12 are represented diagrammatically below the coding sequence (60).
indicating that their slow recovery is a consequence of the effects of gamma radiation. These three strains, while obviously able to repair ionizing radiation-induced damage, appear to be defective in the temporal control of the repair process. This defect could be as simple as the failure of an enzyme to excise DNA damage as rapidly as the parent strain, or it could involve a more complex regulatory network that controls the timing of events associated with damage repair.

Among the forty-three IRS strains identified as ionizing radiation sensitive in Fig. 2.2, forty were found to be recombination proficient as judged by their ability to take up an antibiotic resistance marker. Only IRS26, IRS27, and IRS47 could not be transformed to streptomycin resistance with chromosomal DNA from LS18. Since defects at any stage in the uptake or incorporation of DNA during natural transformation would result in this failure, it is not possible to state with certainty that IRS26, IRS27, and IRS47 are recombination defective. It does, however, seem likely that they are rec mutant strains. Each, in addition to being non-transformable, is also IRS, a characteristic typical of rec mutant strains of _D. radiodurans_ (24, 45). Also, there is no reason to believe that the cell's loss of non-RecA functions related to transformation affects the cell's ability to cope with DNA
damage. *D. radiodurans* is the only species within the Deinococcaceae that is naturally transformable and the other members of this family are as resistant to DNA damage as *D. radiodurans* (41).

Data gathered by this work permits the pol mutations to be ordered within the pol coding sequence. The pol-2 allele is the only one of the three that can be transformed to radioresistance with pPG9, pPG11, and pPG12 making it the most 5' of the pol alleles. Further, chromosomal DNA from pol-2 (IRS 33) and pol-3 (IRS 38) strains cannot transform each other to radioresistance (Table 2). This places their mutations closer to each other than to the mutation found within the pol-1 strain (IRS 7). The three alleles should, therefore, be arranged as follows within the pol coding sequence: 5' pol-2 pol-3 pol-1 3' (Fig. 2.5).

By using natural transformation, sixteen linkage groups were identified among the mutations represented in the IRS strains (Table 2). Strains that could not restore each other to radioresistance were grouped together and assigned a letter designation. The failure of chromosomal DNA from one IRS strain to restore radioresistance to another IRS strain indicates one of two alternative possibilities: i) that the mutations found in the donor and recipient strains affect the same site, or ii) that
these mutations affect different sites closely linked to each other. In either circumstance, restoration of the radioresistant phenotype is prevented but for different reasons. When the mutations of the donor and recipient are at the same site, the wild type sequence needed to replace the recipient's mutation is not present in the donor DNA, making restoration of radioresistance impossible. When the mutations of the donor and recipient are at different but closely linked sites, successful transformation of the recipient with the wild type sequence results in cotransformation with the donor's mutation. The transformants remain IRS because they carry the donor strain's mutation.

Cotransformation of linked markers is expected during natural transformation. In studies conducted with other transformable species, such as Bacillus subtilis, it is assumed that if two markers cotransform they are near enough to each other to be carried on the same DNA fragment and that there is a strong probability that both genes will be incorporated into the chromosome simultaneously during recombination (29). The cotransformation of linked markers in D. radiodurans is remarkable, however, in that the physical distance between markers appears to be very small. This was demonstrated by using the pol mutants. Chromosomal DNA from the pol-1
mutant strain IRS7, unlike DNA from the pol-2 mutant strain IRS33, efficiently transforms pol-3 mutant strain IRS38 to radioresistance, even though the pol-1 mutation is no more than 990 bp from the pol-3 mutation. In other words, the pol-1 allele is not incorporated into the pol-3 recipient at a detectable frequency despite the proximity of these alleles to each other within the pol coding sequence. This indicates that the size of the piece of DNA incorporated into the recipient strain during transformation of this region of the deinococcal chromosome is less than 1,000 bp. In comparison, the average size of the DNA fragment incorporated into B. subtilis during natural transformation has been estimated to be 4,300 bp (16), and markers 3,000 bp from of each other are incorporated with a cotransformation frequency of greater than seventy-five percent (29). The fragment sizes incorporated in Streptococcus pneumoniae and Haemophilus influenzae are even larger, with estimates of 5 kb (23) and 18 kb (16), respectively. Whether this small fragment size is representative of all fragments incorporated during natural transformation in D. radiodurans remains to be determined.
CHAPTER THREE - THE RADIORESISTANCE OF DEINOCOCCUS RADIODURANS: FUNCTIONS NECESSARY TO SURVIVE IONIZING RADIATION ARE ALSO NECESSARY TO SURVIVE PROLONGED DESICCATION

I. Introduction

It is possible to isolate ionizing radiation resistant bacteria, including *D. radiodurans*, from natural microflora without using irradiation by selecting for desiccation resistance (52). This suggests that at least a subset of cellular functions necessary to survive exposure to ionizing radiation are also necessary to survive desiccation. Since dehydration induces DNA damage in bacteria (4, 12, 13), it is likely that the ability to repair DNA damage is one of these functions. To test this possibility, forty-one IRS derivatives of *D. radiodurans* were evaluated for the ability to survive six weeks desiccation. The findings were striking. Every IRS strain was sensitive to desiccation (Fig. 3.1). In addition, it was established that the process of dehydration induced significant DNA damage that accumulated with time. In fact, the number of DNA dsbs formed following six weeks desiccation are comparable to the number generated by 5,200 Gy gamma radiation. From these data, it appears that for *D. radiodurans* ionizing radiation resistance and desiccation resistance are
Fig. 3.1. The effect of six weeks desiccation on the survival of IRS strains of *D. radiodurans*. Values represented are the mean percent survivals of two separate trials, three replicates per trial. Each survival reported is relative to that strain's titer immediately prior to desiccation.
functionally interrelated phenomena, and that by losing the ability to repair ionizing radiation-induced DNA damage, *D. radiodurans* is sensitized to the lethal effects of desiccation. It appears that *D. radiodurans* is an organism that has adapted to dehydration and that its DNA repair capability is a manifestation of that evolutionary adaptation.

II. Materials and Methods

1. Bacterial Strains and Growth Media

Bacterial strains used in this study are listed in Table 3 in the Appendix. All *D. radiodurans* strains were grown at 30°C in TGY broth (0.5 percent tryptone, 0.3 percent yeast extract, 0.1 percent glucose) or on TGY agar (TGY broth containing 1.5 percent agar).

2. Quantifying Desiccation Resistance

Cells from an exponentially growing culture of each strain examined were collected by centrifugation, washed in four volumes of 10 mM MgSO₄, and suspended in an equal volume of 10 mM MgSO₄. A 100 μl aliquot of this suspension was spotted on a sterile glass coverslip, placed inside a sterile petri dish and dried at 25°C in a desiccator over anhydrous CaSO₄, containing a visual indicator. The desiccators were sealed and the dried cultures stored undisturbed at 25°C for six weeks. Relative humidity within the desiccators was measured as
less than five percent using a membrane hygrometer. Samples were revived by washing the cells free of the plate in 1 ml 10 mM MgSO₄ and plating on TGY agar. Plates were placed in a 30°C incubator and scored for survivors five days later.

3. Monitoring the Appearance of DNA Damage by PFGE

The cultures used to prepare chromosomal DNA were concentrated by centrifugation and washed in butanol-saturated 0.5 M EDTA (pH 8.0) to remove the outer membrane and make the cell wall susceptible to lysozyme. Butanol-stripped cells were concentrated by centrifugation, suspended in 0.5 M EDTA and heated to 65°C for 30 minutes. They were then suspended in 0.05 M EDTA, before being embedded in an equal volume of 1.6 percent low-melting-point agarose in 0.05 M EDTA. Agarose plugs containing the embedded cells were soaked overnight at 45°C in a solution containing 1 mg/ml lysozyme dissolved in 0.05 M EDTA (pH 8.0). The lysozyme solution was removed by aspiration and replaced with a 2 mg/ml pronase E solution in 10 mM Tris-HCl, 0.5 M EDTA, and one percent laurylsarcosine (pH 8.0). The agarose plug was soaked in this solution overnight at 45°C. Chromosomal DNA liberated by this treatment was separated by clamped homogeneous electric fields (CHEF) PFGE with 0.5 X TBE at
1.4V/cm² and 12°C with a 10 to 60 second ramp for 22 hours.

III. Results

1. IRS Mutants of *D. radiodurans* Are Also Sensitive to Desiccation

Forty-one IRS derivatives of *D. radiodurans* were evaluated for the ability to survive desiccation. Forty of these strains were derived from *D. radiodurans* 302, a *uvrA1* derivative of wild type *D. radiodurans* R1 (60). The remaining strain, designated rec30, was derived directly from R1 (45). As presented in a proceeding section of this dissertation, strain 302 is as resistant to ionizing radiation as R1, displaying approximately ninety percent survival following exposure to 5,200 Gy gamma radiation when irradiated during exponential-phase growth. The IRS strains are from two- to 300-fold more sensitive to gamma radiation than their parent strains at this dose.

The effect of dehydration on the viability of the IRS strains is illustrated in Fig. 3.1, which shows the mean percent survivals of individual strains desiccated for six weeks. The radioresistant strains R1 and 302 exhibited approximately sixty-three percent survival. In contrast, every IRS strain tested was substantially more sensitive to desiccation (see Chapter Two, Fig. 2.2). Only three of these strains demonstrated greater than ten percent
survival, and the most desiccation-sensitive strains were from 100- to 250-fold less viable than their parents. From these data, it is apparent that \textit{D. radiodurans}' ionizing radiation resistance and desiccation resistance are functionally interrelated phenomena and that by losing the ability to repair ionizing radiation-induced cellular damage, \textit{D. radiodurans} is sensitized to the lethal effects of desiccation.

2. Dehydration Induces DNA Dsbs

It has been reported that there is a substantial reduction in the molecular weight of \textit{D. radiodurans} R1 chromosomal DNA when it is isolated from cultures that have been exposed to a vacuum for twelve days (12). As illustrated in Fig. 3.2, \textit{D. radiodurans} R1 cultures that have been desiccated over CaSO\textsubscript{4} also suffer extensive DNA damage. Intact chromosomal DNA could not be detected in R1 cultures desiccated for six weeks (Fig 3.2, lane 3). Instead, a wide band appeared near the 50-kb size marker, indicating that a large number of DNA dsbs were introduced either during desiccation or upon rehydration of these cultures. Untreated R1 cultures did not show evidence of DNA dsbs (Fig 3.2, lane 2). If it is assumed that the R1 genome is $3 \times 10^7$ bp (22) and that all the fragments in lane 3 are 50 kb or smaller, a minimum of sixty dsbs would be required to generate the pattern observed in lane 3.
Fig. 3.2. The accumulation of chromosomal DNA dsbs in *D. radiodurans* R1 cultures subjected to desiccation as compared to the accumulation of DNA dsbs arising from other *D. radiodurans* R1 culture treatments. Lane 1 and 5 are lambda ladder size standards (Bio-Rad Laboratories, Richmond, CA). The arrow indicates the 48.5 kb marker. Lane 2 is *D. radiodurans* R1 chromosomal DNA prepared from an untreated culture, and lane 3 is R1 chromosomal DNA prepared from a culture that had been desiccated for six weeks. Cultures were desiccated as described earlier. Lane 4 is R1 chromosomal DNA prepared from a culture that had been exposed to 5,200 Gy gamma radiation. Cells were irradiated at 22°C with a $^{60}$Co source at a dose rate of 14.4 Gy/min. Lane 6 is R1 chromosomal DNA isolated from a culture that was heat treated at 52°C for 68 minutes, conditions which define the LD$_{50}$ of *D. radiodurans* R1 as described by Bridges et al. (7). Heat treatment was carried out in a water bath by incubating a thin-walled polypropylene screw-capped tube containing 5 ml of exponential phase cell culture. Lane 7 is R1 chromosomal DNA isolated from a UV-irradiated cell culture. Five ml of exponentially growing cells were concentrated to 500 μl, placed into a disposable petri dish and swirled, uncovered, under a germicidal lamp at a dose rate of 25 J/m$^2$/s until a total dose of 5,000 J/m$^2$ was obtained. Lane 8 is R1 chromosomal DNA isolated from cells held in stationary phase in liquid media at 30°C for 100 days.
The extent of DNA damage observed following desiccation is comparable to what occurs when R1 cultures are exposed to high doses of gamma radiation (Fig. 3.2, lane 4). Other lethal stresses, including heat (Fig. 3.2, lane 5) and starvation during prolonged storage in stationary phase (Fig. 3.2, lane 6), have no obvious effect on the size of chromosomal DNA. Even a massive dose of UV radiation (Fig. 3.2, lane 7) generates only enough damage to produce a faint smear on the gel. Although, we did not formally investigate the formation of desiccation-induced DNA dsbs, the similarities between the gel pattern produced by the chromosomal DNA isolated from desiccated cultures and that isolated from gamma-irradiated cultures suggest that radical chemistry is involved in their formation.

3. Dehydration-Induced DNA Dsbs Accumulate as a Function of Time

The damage inflicted during desiccation accumulates slowly. DNA dsbs are not obvious in D. radiodurans cultures until eight days after desiccation (Fig. 3.3). Beyond twenty-eight days it is difficult to detect intact chromosomal DNA, and by forty-two days only low-molecular weight DNA is apparent. Cultures of the E. coli K-12 strain AB1157 also accumulate DNA dsbs with the same kinetics as D. radiodurans (Fig. 3.4), suggesting that the DNA dsbs observed when D. radiodurans is dried are solely
Fig. 3.3. The appearance of DNA dsbs in a desiccated *D. radiodurans* R1 culture as a function of time. Lane 1 is a lambda ladder size standard. The arrow indicates the 48.5 kb marker. Lane 2 is *D. radiodurans* R1 chromosomal DNA prepared from an untreated culture. Lanes 3-8 are R1 chromosomal DNA preparations obtained from cultures after 1, 4, 8, 14, 28, 42 days of desiccation, respectively.

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Fig. 3.4. The appearance of DNA dsbs in a desiccated *E. coli* AB1157 culture as a function of time. Lane 1 is a lambda ladder size standard. The arrow indicates the 48.5 kb marker. Lane 2 is AB1157 chromosomal DNA prepared from an untreated culture. Lanes 3–8 are AB1157 chromosomal DNA preparations obtained after 2, 5, 16, 28, 42 days of desiccation, respectively.
a consequence of desiccation, and not a function of physiological changes peculiar to *D. radiodurans*.

4. At Least One Other Factor Also Influences the Ability of *D. radiodurans* to Survive Dehydration

The survival curves of the *E. coli* and *D. radiodurans* cultures used to generate Figs. 3.3 and 3.4 are plotted in Figure 3.5. The viability of the *E. coli* cultures declined dramatically upon desiccation. There was a 1,000-fold reduction in survivors by the second day of desiccation, and by day fourteen it was not possible to detect viable organisms in the dried *E. coli* cultures; that is, there was less than one viable organism per 100 μl of original culture. The rapid loss of viability in the dried *E. coli* cultures was not accompanied by overt evidence of DNA dsbs (Fig. 3.4), indicating that either multiple DNA dsbs were not responsible for the observed lethality or more DNA material is found within *D. radiodurans* multigenomic cells (up to ten copies in exponential phase in *D. radiodurans* cells (43) than in *E. coli* cells).

There was no loss of viability in *D. radiodurans* cultures during the first fourteen days of desiccation. Over the next twenty-eight days, however, there was an 11-fold reduction in viability, a value inconsistent with data presented in Fig. 3.1 A where sixty-three percent...
Fig. 3.5. Representative survival curves for *D. radiodurans* R1 (squares) and *E. coli* AB1157 (circles) as a function of time spent desiccated over CaSO₄. Values are the means of a single trial, three replicates.
survival was reported in a nearly identical experimental protocol. The *D. radiodurans* R1 cultures used to generate Fig. 3.5 exhibited only seventeen percent survival after six weeks desiccation. The two experiments differed only in the numbers of times the desiccator was opened during each trial. The desiccator was sealed throughout the six weeks of desiccation during the studies represented in Figs. 3.1 and 3.2, but it was opened at each time point examined in Figs. 3.3 and 3.4. This suggested that periodic opening of the desiccator was harmful to the dried cells.

To evaluate this possibility, the experiment described in Fig. 3.1 was repeated, but the desiccator was opened for thirty minutes at the beginning of each week of the six-week trial. Survival of the R1 and 302 strains (Fig. 3.6) was reduced to thirteen percent when this modified protocol was followed. This value is consistent with the seventeen percent survival reported in Fig. 3.5. The IRS strains exhibited a similar reduction in survival, but they remained between ten- and 100-fold less viable than their parent strains when rehydrated. The physical basis of this effect was not investigated, but it was noted that as the desiccator was opened the relative humidity within the system increased to approximately sixty percent and, upon resealing the system, it took from
Fig. 3.6. The effect of opening of the desiccator on the survival of IRS strains of *D. radiodurans*. Values represented are the mean percent survivals of four separate trials, three replicates per trial. Cultures were desiccated as described except that at the beginning of each week in the six week trial the desiccator was opened to ambient humidity for thirty minutes.
thirty to forty-five minutes for it to return to a value less than five percent. It is, therefore, possible to lower the survival of desiccated cultures of *D. radiodurans* by subjecting them to cycles of desiccation and partial rehydration.

5. IRS Strains Are Unable to Repair Desiccation-induced DNA Damage

A subset of the IRS strains are unable to adequately repair desiccation-induced DNA damage when observed by PFGE, whereas the parent strain, *D. radiodurans* 302, can (Fig. 3.7). The parent strain recovers from desiccation nine hours post-rehydration as intact chromosomal material, the high-molecular-weight band, is present at this time (Fig. 3.7, lane 5). Two of the IRS strains were arbitrarily observed. These strains, IRS41 and IRS18, do not exhibit an intact chromosomal band after nine hours post-rehydration (Fig. 3.7, lanes 9 and 13, respectively).

IV. Discussion and Conclusions

The ionizing radiation resistance of *D. radiodurans* has been difficult to explain from an evolutionary point of view, because the selective advantage of this extreme character is not obvious. The average outdoor terrestrial absorbed dose rate in air from gamma radiation is $5 \times 10^{-8}$ Gy h$^{-1}$ or 0.4 mGy y$^{-1}$ and the highest reported absorbed dose rate, measured in thorium-rich monazite sands found near
Fig. 3.7. Evidence for the repair of desiccation-induced DNA damage in wild type *D. radiodurans* R1, but not in IRS mutants. Lanes 2-5 represent repair of DNA in a desiccated and revived *D. radiodurans* R1 culture after recovery times of 0, 3, 6, and 9 hours respectively. Lane 1 is untreated DNA from R1. Lanes 6-9 are a desiccated and rehydrated IRS41 culture at 0, 3, 6, and 9 hours post-rehydration, respectively. Lanes 10-13 are as for IRS41, but with IRS18.
Guarapari, Brazil, is only 175 mGy y⁻¹ (62), far too low to be considered the selective force that could build the degree of radioresistance associated with wild-type *D. radiodurans*. It has therefore been assumed that the radiotolerance of *D. radiodurans* is a fortuitous consequence of an evolutionary process that permitted this bacterium to cope with an environmental stress other than ionizing radiation.

In this study, it is established that *D. radiodurans*’ cellular responses to ionizing radiation and dehydration significantly overlap by showing that forty-one IRS strains of *D. radiodurans* are also sensitive to desiccation (Fig. 3.1). It is inferred that the loss of DNA repair capability in the IRS strains is responsible for the desiccation sensitivity of these strains. Four interrelated arguments support this inference. First, prolonged dehydration causes extensive DNA damage (Figs. 3.2 and 3.3), with six weeks of desiccation resulting in a minimum of sixty DNA dsbs, indicating that to survive desiccation, the *D. radiodurans* cell requires extraordinary DNA repair capability. Second, there is evidence provided from studies of *E. coli* that desiccation resistance is influenced by the DNA repair capability of the strain being dehydrated. Asada et al. (4) have shown that AB2480, a recA uvrA isogenote of AB1157, is
substantially more sensitive to drying under a vacuum than is AB1157. We assume that, if DNA repair capability contributes to the survival of a desiccation sensitive organism such as *E. coli*, then it should contribute to the survival of a desiccation resistant organism as well.

Third, nine of the strains represented in Fig. 3.1 express characterized DNA repair defects. IRS26, IRS27, IRS47 and rec30 are recombination-defective mutants as presented in Chapter Two. As shown earlier in this dissertation, IRS7, IRS33, and IRS38 express inactive forms of the deinococcal homolog of *E. coli*’s DNA polymerase I, and genetic evidence indicates that IRS18 and IRS41 express proteins unable to appropriately regulate repair of DNA damage. For these strains, the loss of DNA repair capability resulted in sensitivity to desiccation. Finally, IRS strains are apparently unable to repair desiccation-induced DNA damage (Fig. 3.7).

The role of DNA repair in the desiccation resistance of *D. radiodurans* is similar to that ascribed for DNA repair in *Bacillus* spores (53). During their dormancy, spores accumulate DNA damage that cannot be repaired until the spores germinate. The lack of water within the spore prevents enzymatic activity and therefore DNA repair. As demonstrated in Figs. 3.3 and 3.4, there is the potential for DNA damage to accumulate in vegetative cells during
Desiccation. It is assumed that desiccation-induced DNA damage cannot be repaired until the organism is rehydrated. Desiccation-resistant vegetative organisms must either be able to inhibit the formation of DNA damage while dehydrated or repair it upon rehydration. In *D. radiodurans*, it is the capacity to repair DNA damage which appears most developed.

In *E. coli*, dehydration-induced lethality (Fig. 3.5) did not appear to correlate with the formation of DNA dsbs (Fig. 3.4), suggesting that the differences between *D. radiodurans* and *E. coli* extend beyond the ability of each cell to repair DNA damage. This was not unexpected, since dehydration adversely affects the membraneous and proteinaceous components of the cell as well as its nucleic acids (51). One would predict that *D. radiodurans*, being desiccation resistant, has mechanisms that contend with or inhibit all dehydration-induced cellular damage and that *E. coli* would not have such mechanisms. Pending further investigation, we assume that the loss of viability observed when the desiccator is repeatedly opened is either due to an increase in DNA damage caused by the cycles of dehydration or to the disruption of a part of *D. radiodurans'* defense against dehydration that is not related to DNA damage repair.
It is concluded that *D. radiodurans* is an organism that has adapted to dehydration and that its DNA repair capability is a manifestation of that evolutionary process. *D. radiodurans* is ionizing radiation resistant because it is resistant to desiccation and because desiccation resistance appears to require extensive DNA repair.
I. Introduction

*D. radiodurans* cell cultures respond to irradiation by a general shut down of growth and DNA synthesis, during which DNA damage removal and repair occurs (11, 34, 43). Repair of both UV and ionizing radiation induced DNA damage is characterized by a degradation process which proceeds at a constant rate, continuing for a length of time determined by the amount of radiation exposure received by the cells (15, 20, 36, 43). Following UV exposure, nucleotides are exported into the surrounding media (43, 64). The export of nucleotides has been shown to correspond to the repair of DNA base damage, specifically thymine dimers (43). Following ionizing radiation exposure, nucleotides are also exported into the surrounding media. Additionally, density gradient centrifugation and PFGE show that a genome exposed to ionizing radiation contains multiple sites of double-strand DNA breakage (43). During the repair process an increase in the size of the isolated DNA is observed, indicating that the broken strands of DNA are reassembled into an intact chromosomal state (43, 64). Following UV exposure DNA replication and cell growth resume after nucleotide export ceases (43, 64). Following ionizing
radiation exposure, cell growth resumes after nucleotide export ceases, and either during or shortly after DNA strand breaks are mended (43, 64). Although the responses to radiation are well documented in *D. radiodurans*, the enzymatic features underlying these responses to radiation have remained poorly understood.

A cosmid library of the *D. radiodurans* R1 genome was used to restore the radiation resistant phenotype to some of the sixteen linkage groups comprising the IRS mutant collection (60). As a result, several novel genetic loci involved in ionizing radiation survival have been identified. One locus was identified in group I of the IRS mutant collection and has been designated *irrl* (60). UV and ionizing radiation survival data gathered for *irrl* mutants show that the presence of the *irrl* mutation affects survival post-irradiation depending upon i) the presence or absence of a functional *uvrA* locus and ii) the type of radiation administered (60). PFGE techniques (22) are utilized here to further examine the responses of an *irrl* and an *irrl uvrA* mutant to irradiation. Analysis of DNA repair kinetics and cell survival during a post-irradiation time course allow speculation upon the role of the *irrl* gene product in *D. radiodurans'* response to irradiation.
II. Materials and Methods

1. Bacterial Strains and Growth Media

Bacterial strains used in this study are listed in Table 3 in the Appendix. All *D. radiodurans* strains were grown at 30°C in TGY broth (0.5 percent tryptone, 0.3 percent yeast extract, 0.1 percent glucose) or on TGY agar (TGY broth containing 1.5 percent agar).

2. Gamma Irradiation of Cells Prior to PFGE Analysis

Cell samples were placed into a microcentrifuge tube and exposed to a $^{60}$Co source at an exposure rate of 14.4 Gy/minute until the desired dose was achieved. Cells were then immediately used for DNA isolation.

3. Procedure for Estimation of CFU and for Obtaining Cell Samples for DNA Isolation during a Time Course of Repair Post-irradiation

Six milliliters of eighteen hour *D. radiodurans* cell cultures were concentrated to 1.5 ml. A 200 μl control aliquot was removed. Ten microliters of this aliquot were serially diluted in 10 mM MgSO$_4$ to determine the number of CFU/ml in the sample. The remaining 190 μl of the aliquot was used for DNA isolation. The remainder of the original 1.5 ml culture was exposed to radiation in 1 x TGY broth or in 10 mM MgSO$_4$ for recovery experiments. Ten mM MgSO$_4$ was chosen as the non-nutritive, isotonic solution, because it has no observable effect upon *D. radiodurans*
cell cultures. Sodium chloride causes *D. radiodurans* cells to produce membraneous bodies, manganese ions have been reported to cause reductive division in some mutant strains, and calcium ions affect DNA uptake by the cells (31, 43). Immediately after irradiation, four 200 μl aliquots of these cells were taken. One aliquot was immediately titered, pelleted, and used for DNA isolation at time-zero. The other three 200 μl aliquots were diluted 1:11 individually by adding them to three 2 ml tubes of fresh 1 x TGY broth or 10 mM MgSO₄. The cells were incubated with constant shaking at 30°C to allow recovery from radiation exposure. One of the tubes was withdrawn every three hours post-irradiation. The contents of each tube was titered, and the remaining contents of each tube pelleted and used for DNA isolation. This procedure is represented diagrammatically in Figure 4.1.

4. DNA Isolation

Cell samples were pelleted at 4000 x g for five minutes immediately following treatment, if any. They were rinsed with 0.5 ml butanol-saturated 0.5 M EDTA (pH 8.0), thoroughly suspended, and pelleted. The cells were suspended immediately in 0.5 ml 0.5 M EDTA (pH 8.0) and heat treated for thirty minutes at 68°C to inactivate nuclease. All of the supernatant was removed and the
6.0 ml concentrated to 1.5 ml

CONTROL 200-μl aliquot removed, titered, and used for DNA isolation

remaining contents irradiated

τ=0 200-μl aliquot removed, titered, and used for DNA isolation

3 200-μl aliquots removed, each added to fresh 2-ml tubes of growth media or buffer, and incubated at 30°C with constant shaking

τ=3 titered, and used for DNA isolation

τ=6 titered, and used for DNA isolation

τ=9 titered, and used for DNA isolation

Fig. 4.1. Diagrammatic representation of the procedure for estimation of CFU/ml and for obtaining cells for DNA isolation during a time course of repair.
cell pellet was suspended in 100 μl of 0.05 M EDTA (pH 8.0). One-hundred μl of cooled 1.6 percent low-melting-point agarose in 0.05 M EDTA was immediately added to the cell solution. This solution was aspirated into sterile tubing (1.58 mm diameter). The mixture in the tubing was allowed to solidify at room temperature for fifteen minutes and then was expelled into a screw-capped tube containing 0.5 ml of lysis solution (0.05 M EDTA containing 2 mg/ml lysozyme). The cells were incubated at 45°C overnight. The next day, the solution was removed from around the agarose embedded DNA sample and replaced with 0.5 ml NDS solution (0.5 M EDTA, pH 8.0, 10 mM Tris-HCl, pH 7.5, one percent N-lauroylsarcosine, 4 units/ml proteinase K). The sample was allowed to incubate overnight at 45°C. Samples were stored at 4°C in 1 ml of 0.05 M EDTA (pH 8.0) or used immediately.

5. PFGE of DNA Samples

Agarose-embedded DNA samples were cut into 1 cm pieces, pre-soaked in 0.5 X TBE buffer, and loaded into a one percent agarose gel made with 0.5 X TBE buffer. A CHEF-DR II apparatus (Bio-Rad Laboratories, Richmond, CA) was used to separate the DNA. CHEF-DR II parameters were 14 V/cm, 12°C, with a 10 to 60 second ramp for 22 hours.
III. Results

1. The *irrI* Mutation Slows Reassembly of the Chromosomal Band Following Ionizing Radiation Exposure

PFGE allows DNA damage and repair to be qualitatively evaluated based upon the presence or absence of dsbs and an intact chromosomal band. DNA isolated from untreated *D. radiodurans* cells is characterized by the presence of a single, high molecular weight band that is the intact chromosome. Following administration of radiation, isolated DNA may exhibit dsbs. Dsbs may occur as a result of direct breakage of the DNA backbone or may also occur as a result of cellular enzymatic activity necessary to repair DNA damage sites. Dsbs present in an isolated DNA sample are visible on a gel as a smear of lower molecular weight DNA fragments. Repair of dsbs is evident when this smear is replaced by a chromosomal band over a time course of recovery. The addition of a protein denaturing step to the DNA isolation process for PFGE made it possible to reproducibly observe *D. radiodurans* cell samples at defined time points following irradiation. Cells were exposed to radiation and observed by cell survival measurements and PFGE. Recovery was carried out by diluting freshly irradiated cells in growth media. Observations were taken immediately post-irradiation and for a total of nine hours at three hour intervals.
Two types of *irrI* mutants were observed: IRS41, an *irrI uvrA* double mutant, and IRS411, an *irrI* mutant. Observations made of the *irrI* and *irrI uvrA* mutants were compared to results seen with two control strains, wild type strain R1 and strain 302, the *uvrA* parent strain of the *irrI* mutants.

Following exposure to 3,450 Gy gamma radiation, dsbs are observed immediately by PFGE analysis in all strains (Fig. 4.2). The chromosomal band is observed three hours post-irradiation in wild type strain R1 and nine hours post-irradiation for the *uvrA* strain (Fig. 4.2 A and B). The chromosomal band is not seen during the nine hour recovery period for the *irrI* and *irrI uvrA* mutants (Fig. 4.2 C and D). Cell survival was monitored during the recovery time course. This allowed the success of repair processes to be appraised, as any display of cell division could be assumed to correlate with the successful completion of DNA repair. Cell survival data with the wild type and *uvrA* strains indicated that cell division was occurring within nine hours after exposure to gamma radiation. Survival figures show the CFU/ml (solid circles) at each recovery time point following gamma irradiation compared to the CFU/ml obtained from unirradiated control cultures (dashed line). Wild type (Fig. 4.3 A) and *uvrA* (Fig. 4.3 B) cultures irradiated

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Fig. 4.2. PFGE analysis of *D. radiodurans* control strains and *irrI* mutants exposed to 3,450 Gy gamma radiation and recovered in growth media. A) strain R1 (wild type). B) strain 302 (*uvrA*). C) IRS 41 (*irrI uvrA*). D) IRS411 (*irrI*). In all gels the lanes are as follows: lane 1, lambda ladder size standard; lane 2, unirradiated control; lanes 3-6, chromosomal DNA preparations obtained 0, 3, 6, and 9 hours after irradiation.
Fig. 4.3. Recovery post-irradiation of A) wild type and B) uvrA strains exposed to 3,450 Gy gamma radiation and incubated in growth media. The dashed line represents the number of CFU/ml in the unirradiated control.
with a dose of 3,450 Gy displayed survivals near 100 percent at time zero. Both cultures remained completely viable during the nine hour recovery period. The irrI uvrA (Fig. 4.4 A) mutant and the irrI (Fig. 4.4 B) mutant exhibited two percent survival and less than one percent survival, respectively, immediately after exposure to 3,450 Gy. Both showed a decrease in survival during the nine hour recovery period.

2. The irrI Mutant Loses Chromosomal DNA Following UV Exposure

Following administration of 500 J/m² UV radiation, dsbs are not detectable in the wild type, uvrA, and irrI uvrA strains (Fig. 4.5 A, B and C). This was expected, since UV radiation does not normally induce DNA dsbs. However, a decrease in the amount of intact chromosomal material is present at the three hour recovery interval in the irrI mutant (Fig. 4.5 D). This suggests that the irrI mutant possesses a defect allowing cellular enzymatic activity to convert UV radiation-induced lesions into dsbs. The irrI mutant is more sensitive to the UV dose than the wild type, uvrA, or the irrI uvrA strains (Figs. 4.6 and 4.7).

All strains and mutants show an increase in cell numbers during the recovery time course. The increase in
Fig. 4.4. Recovery post-irradiation of A) irrI uvrA and B) irrI mutants exposed to 3,450 Gy gamma radiation and incubated in growth media. The dashed line represents the number of CFU/ml in the unirradiated control.
Fig. 4.5. PFGE analysis of A) strain R1 (wild type), B) strain 302 (uvrA), C) IRS41 (irrI uvrA), and D) IRS411 (irrI) exposed to 500 J/m² UV radiation and recovered in growth media. In all gels the lanes are as follows: lane 1, lambda ladder size standard; lane 2, unirradiated control; lanes 3-6, chromosomal DNA preparations obtained 0, 3, 6, and 9 hours after UV exposure, respectively.
Fig. 4.6. Recovery post-irradiation of A) wild type and B) uvrA strains exposed to 500 J/m² UV radiation and incubated in growth media. The dashed line represents the number of CFU/ml in the unirradiated control.
Fig. 4.7. Recovery post-irradiation of A) *irrI uvrA* and B) *irrI* mutants exposed to 500 J/m² UV radiation and incubated in growth media. The dashed line represents the number of CFU/ml in the unirradiated control.
cell numbers during the recovery period for all strains and mutants indicated a potential problem. The possibility existed that the reappearance of the chromosomal band could be due to the outgrowth of a surviving subset of the population and not entirely due to reassembly of the chromosomal band. For example, $1.5 \times 10^5$ CFU/ml doubling every eighty minutes would become approximately $4.5 \times 10^5$ CFU/ml in three hours, $3.6 \times 10^6$ CFU/ml in six hours, and $9.6 \times 10^6$ CFU/ml in nine hours. Therefore, an outgrowth effect could mask important observations concerning the fate of non-recovering cells.

Results gathered at zero and three hours post-irradiation would be far more accurate than results gathered at six and nine hours post-irradiation. This could account for the outgrowth post-irradiation that is seen with the *irrI* mutant (Fig. 4.5 D, lanes 4 - 6) in which the chromosomal band reappears even though cell survival was only two percent at time-zero post-irradiation.

Outgrowth could, however, be stopped if cell cultures were not allowed to recover in growth media. Therefore, cultures were UV irradiated in a magnesium sulfate solution instead of growth media and observed as before.
3. Only the *irrI* Mutant Exhibits Enhanced DNA Degradation Following UV and Ionizing Radiation Exposure

The results obtained when the cells are allowed to recover in 10 mM MgSO₄ show that the outgrowth problem is eliminated. Viability for the wild type and *uvrA* control strains following UV radiation exposure does not increase significantly during incubation in 10 mM MgSO₄ solution for eighteen hours (Figs. 4.8 and 4.9). The *irrI* *uvrA* mutant (Fig. 4.10), although it does exhibit some sensitivity to UV radiation, does not show a significant decrease in viability during the incubation period and does not display the dramatic sensitivity to UV seen in the *irrI* mutant.

The *irrI* mutant, however, is extremely UV radiation-sensitive (Fig. 4.11). Viability was not detectable three hours after UV exposure. The *irrI* mutant's extreme sensitivity to radiation is explainable after PFGE examination. Immediately following UV radiation exposure, the *irrI* mutant's DNA remains intact as shown by the high molecular weight DNA band seen in lane 3 (Fig. 4.12 B). However, the DNA completely disappears during subsequent hours of incubation (Fig. 4.12 B, lanes 4 - 6), perhaps by degradation. Wild type (Fig. 4.13 A), *uvrA* (Fig. 4.13 B), and *irrI* *uvrA* (Fig. 4.12 A) cells show no evidence of DNA
Fig. 4.8. Recovery post-irradiation of wild type strain R1 exposed to A) 500 J/m² UV and B) 3,450 Gy gamma radiation and incubated in 10 mM MgSO₄. The dashed line represents the number of CFU/ml in the unirradiated control.
Fig. 4.9. Recovery post-irradiation of uvrA strain 302 exposed to A) 500 J/m² UV and B) 3,450 Gy gamma radiation and incubated in 10 mM MgSO₄. The dashed line represents the number of CFU/ml in the unirradiated control.
Fig. 4.10. Recovery post-irradiation of the *irrI uvrA* mutant exposed to A) 500 J/m² UV and B) 3,450 Gy gamma radiation and incubated in 10 mM MgSO₄. The dashed line represents the number of CFU/ml in the unirradiated control.
Fig. 4.11. Recovery post-irradiation of the irrI mutant exposed to A) 500 J/m² UV and B) 3,450 Gy gamma radiation and incubated in 10 mM MgSO₄. Recovery was not detectable following UV exposure. Recovery was not detectable after six hours recovery following ionizing radiation exposure. The dashed line represents the number of CFU/ml in the unirradiated control.
Fig. 4.12. PFGE analysis of the A) irrA uvrA and B) irrI mutants exposed to UV and gamma radiation and incubated in 10 mM MgSO₄. In both gels the lanes are as follows: lane 1, lambda ladder size standard; lane 2, unirradiated control; lanes 3-6, chromosomal DNA preparations obtained 0, 3, 6, and 9 hours after a dose of 500 J/m², respectively; lanes 7-10, chromosomal DNA preparations obtained 0, 6, 12, and 18 hours after gamma radiation exposure.
Fig. 4.13. PFGE analysis of the A) wild type and B) uvrA strains exposed to UV and gamma radiation and incubated in 10 mM MgSO4. In both gels the lanes are as follows: lane 1, lambda ladder size standard; lane 2, unirradiated control; lanes 3-6, chromosomal DNA preparations obtained 0, 3, 6, and 9 hours after a dose of 500 J/m², respectively; lanes 7-10, chromosomal DNA preparations obtained 0, 6, 12, and 18 hours after gamma radiation exposure.
degradation following UV exposure, and the chromosomal band is maintained (lanes 3 - 6).

Following ionizing radiation exposure, dsbs are present in all strains (Figs. 4.12 and 4.13, lane 7). Although these dsbs do not appear to undergo repair into a complete chromosomal band after an eighteen hour incubation period in 10 mM MgSO₄, the fragments appear to remain "stabilized" or "held" during the entire incubation period in all strains except the irrI strain (lanes 8 - 10). The irrI uvrA mutant does show the same initial survival as seen when allowed to recover in growth media. A decrease in viability for this strain is not seen over time as when the cells are allowed to recover in growth media.

4. Reappearance of a Chromosomal Band Post-irradiation Requires Incubation Under Conditions Allowing Growth

After dsbs have been introduced into the chromosome, complete repair of the chromosome seems to require conditions which allow growth. This is evident from the 10 mM MgSO₄ recovered gamma-irradiated PFGE results with the wild type and uvrA strains (Fig. 4.13, lanes 7 - 10). In these strains, the chromosomal band does not reappear after eighteen hours of incubation in 10 mM MgSO₄, even though viability remains at 100 percent. This is unlike the reappearance of the chromosomal band seen under the
same conditions after nine hours incubation in fresh
growth media (Fig. 4.2 A and B).

IV. Discussion and Conclusions

Following UV and ionizing radiation exposure with
recovery in 10 mM MgSO₄, the irrI mutation in a uvrA⁻
background clearly appears to allow DNA degradation to
continue unchecked, resulting in the complete
disappearance of DNA from the gel (Fig. 4.12 B) and in
cell death (Fig. 4.11). In contrast, the irrI mutation in
conjunction with a uvrA mutation does not exhibit DNA
degradation when examined by PFGE (4.12 A). Instead, the
DNA fragments appear to be "held" until growth conditions
are present, as is seen in the control strains. The irrI
uvrA mutant shows sensitivity at time zero, but survival
does not decrease during the incubation period (Fig.
4.10), as is seen with the irrI mutant.

This data indicates that the irrI gene product acts
as an inhibitor of DNA degradation initiated by the UvrA
protein. Inhibition may act directly upon UvrA, upon an
inhibitor required to shut down activities associated with
the UvrA protein, or upon DNA degradation enzymes present
in D. radiodurans. UvrA may identify DNA damage for
subsequent degradation or may both identify and degrade
DNA at damage sites. The irrI gene product appears to
inhibit degradation at DNA damage sites exhibiting DNA
strand breakage. Following UV exposure the UvrA-protein would identify DNA base damage. This damage would probably be excised leaving a single strand gap. The irrI gene product would then prevent DNA degradation at this gap until the gap could be repaired. In the absence of the irrI gene product DNA degradation would proceed unchecked at gap sites, leading to cell death. Similarly, in the absence of the irrI and the uvrA gene product, this effect would be avoided, as UvrA would be unavailable to form the gaps from damaged DNA. Following ionizing radiation exposure, the ionizing radiation itself would introduce single-strand gaps and double-strand breaks into the DNA. DNA degradation would proceed unchecked in the absence of the irrI gene product. The presence of the UvrA protein would only further increase the chances for DNA degradation as DNA base damage arising from ionizing radiation exposure would be converted into gaps available for subsequent degradation. This explains the increased sensitivity of the irrI mutant to ionizing radiation, relative to the irrI uvrA mutant. The data also indicate that the UvrA protein is an active component in both the UV and the ionizing radiation repair mechanism. uvrA mutants, although not detectably more sensitive to ionizing radiation than wild type cells at the dose used in these experiments, do appear to require a longer
recovery time to display an increase in survival than does the wild type.

It is not clear whether DNA replication or cellular growth is required for completion of the dsb repair process, although a few reports indicate that recovering cells must be put in fresh growth media or the repair process cannot entirely proceed (36, 37). The data indicate that cells must recover in conditions allowing growth for completion of the repair process, i.e. the appearance of the chromosomal band. In Fig. 4.13, lanes 7 - 10, PFGE results following ionizing radiation exposure show that in the wild type and non-IRS uvrA strains incubation in 10 mM MgSO₄ solution does not allow the chromosomal band to reappear after dsbs have been introduced.
CHAPTER FIVE - CONCLUSION

The extreme radioresistance of the Deinococcaceae Family has generated many long standing questions in the minds of those who have researched and studied these organisms. The research carried out in this dissertation upon \textit{D. radiodurans} has provided an answer to one of the most persistent questions regarding extreme radioresistance, specifically, the question of how extreme radioresistance may have evolved under natural conditions. By examining a collection of IRS mutants, a correlation between desiccation sensitivity and ionizing radiation sensitivity was established. The use of PFGE allowed another correlation to be made concerning the prevalence of DNA dsbs in both desiccated and ionizing radiation exposed cells. Because desiccation and ionizing radiation exposure caused a similar DNA lesion, the dsb, and because mutants unable to repair ionizing radiation damage were also unable to repair desiccation damage, an important conclusion could be made: \textit{D. radiodurans} was able to survive ionizing radiation damage because it could survive desiccation damage. Therefore, the radioresistance mechanisms of \textit{D. radiodurans} could have evolved in response to the DNA damage incurred during periods of desiccation.
Research presented in this dissertation also expands the ability to study *D. radiodurans*. The development of the dot transformation technique has facilitated the ability to screen mutant collections, cosmid libraries, and any cloned gene of interest in this organism. The data presented in Chapter Two of this dissertation was acquired by the use of the dot transformation technique. The collection of IRS mutants was screened and organized into linkage groups. In particular, information provided by the screen for *pol* mutations (60) in conjunction with the linkage group information revealed that the distance between cotransformed markers in *D. radiodurans* is remarkably small.

By using PFGE to observe mutants within the IRS collection, the function of the deinococcal gene product designated *IrrI* has been roughly defined. Data show that this product is involved in preventing DNA degradation during the repair of UV and ionizing radiation-induced DNA damage. The observation of *irrI* mutant behavior is the first study carried out on a novel mutant of *D. radiodurans*.

This dissertation serves to demystify *D. radiodurans*. It provides an answer to the origin of extreme radioresistance, defines a previously unknown function for the enzymes utilized in radioresistance by observing the
novel *irrI* and *irrI uvrA* mutants, and provides an important tool, dot transformation, for the study of this organism.
BIBLIOGRAPHY


## APPENDIX

<table>
<thead>
<tr>
<th>Bacterial Strain or plasmid</th>
<th>Relevant description</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td><em>Deinococcus radiodurans</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R1</td>
<td>ATCC 13939</td>
<td>(3)</td>
</tr>
<tr>
<td>302</td>
<td>As R1 but <em>uvrA</em></td>
<td>(41)</td>
</tr>
<tr>
<td>rec30</td>
<td>As R1 but <em>rec</em></td>
<td>(45)</td>
</tr>
<tr>
<td>IRS1-IRS49</td>
<td>As 302 but IRS</td>
<td>(60)</td>
</tr>
<tr>
<td>IRS26, IRS27, IRS47</td>
<td>As 302 but nontransformable</td>
<td>(60)</td>
</tr>
<tr>
<td>IRS7, IRS33, IRS38</td>
<td>As 302 but <em>pol</em></td>
<td>(60)</td>
</tr>
<tr>
<td>IRS181</td>
<td>As IRS18 but <em>uvrA</em>'</td>
<td>(60)</td>
</tr>
<tr>
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<td>As IRS41 but <em>uvrA</em>'</td>
<td>(60)</td>
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<td>SLR1</td>
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</tr>
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<td>this study</td>
</tr>
<tr>
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<td>this study</td>
</tr>
<tr>
<td>SLR5</td>
<td>Formerly IRS17</td>
<td>this study</td>
</tr>
<tr>
<td>LS18</td>
<td>Same as R1 but streptomycin resistant</td>
<td>(60)</td>
</tr>
</tbody>
</table>

*(table con'd.)*
Escherichia coli

AB1157
\[\text{thr-1 \ ara-14 \ leuB6} \ \Delta \text{(gpt-proA)62 \ lacY1 \ tsx-33 \ galK2} \]
\[\text{his64 (oc) \ rfbD1 \ mgl-1} \]
\[\text{argE3 \ thi-1; \ wild \ type} \]
\[\text{levels \ of \ UV \ and \ ionizing \ radiation \ resistance} \]

DH5α-MCR
\[\text{F}^{-} \ \text{mcrA} \ \Delta \text{(mrr-hsdRMS-mcrBC) \ \Phi80 \ lacZ \ dm15} \]
\[\Delta \text{(lacZYA-argF) \ U169 \ endA1} \]
\[\text{deoR \ thi-1 \ supE44 \ \lambda- gyrA96} \]
\[\text{relA1; \ wild \ type \ levels \ of} \]
\[\text{UV \ and \ ionizing \ radiation \ resistance} \]

Plasmids

pPG9
\[\text{pBC SK'} \ (\text{Stratagene, La Jolla, CA}) \ \text{derivative} \]
\[\text{with} \]
\[2.3-\text{kb \ insert \ of} \]
\[\text{D. radiodurans \ genomic \ DNA} \]
\[\text{carrying} \ 1.9-\text{kb \ fragment} \]
\[\text{of} \]
\[\text{pol \ gene} \]

pPG11
\[\text{pBluescript SK'} \ (\text{Stratagene, La Jolla, CA}) \]
\[\text{derivative} \]
\[\text{with} \]
\[5.2-\text{kb \ insert \ of} \]
\[\text{D. radiodurans} \]
\[\text{genomic \ DNA \ carrying} \ 1.89-\text{kb \ fragment \ of} \]
\[\text{pol \ gene} \]

pPG12
\[\text{pBC SK'} \ \text{derivative} \]
\[\text{with} \]
\[1.2-\text{kb \ insert \ of} \]
\[\text{D. radiodurans} \]
\[\text{genomic \ DNA} \]
\[\text{carrying} \ 0.9-\text{kb \ fragment} \]
\[\text{of} \]
\[\text{pol \ gene} \]
VITA

Valerie Mattimore was born on December 22, 1967 in Lynwood, California, a suburb of Los Angeles. She attended five kindergartens, three elementary schools, and two junior high schools all over the United States before attending Sapulpa High School in Sapulpa, Oklahoma. (Uh, its near Tulsa....uh, Tulsa is near, uh,...huh, huh,...) She graduated from Sapulpa High School in 1986 as the valedictorian. She then went to Oklahoma State University where she did not pledge a Greek society, did major in Microbiology, and did lose all ambition. After two years she transferred to the University of Tulsa to major in Biology-PreMed. She graduated from the University of Tulsa in May, 1990, with a bachelor of science degree in Biology and an intense loathing for anal-retentive PreMed students. She married a Turk that same month. She then entered the Graduate School at Louisiana State University in August of the same year. Six long years, one more marriage, and two divorces later she earned her doctor of philosophy degree in Microbiology. She now plans to open a Jam n' Dine Big-Coffee-Shop-In-The-Sky and sing the blues. Wrote a song about it. Like t' hear it? Here it goes...
DOCTORAL EXAMINATION AND DISSERTATION REPORT

Candidate: Valerie Mattimore

Major Field: Microbiology

Title of Dissertation: The Radioresistance of Deinococcus radiodurans

Approved:

[Signatures]

Major Professor and Chairman

Dean of the Graduate School

EXAMINING COMMITTEE:

[Signatures]

Michael Arntzen
Patrick J. DeMarco
Kathleen Reilly
Beán P. Con-Gynn

Date of Examination:

12/16/96