Analysis of *Deinococcus radiodurans* Mutants

Diplomarbeit

by

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Dedicated to my parents
Ursula and Manfred Hess

Abstract

The extremely radiation resistant vegetative bacterium *Deinococcus radiodurans* was isolated in 1956 from gamma-irradiated meat. Since then, it has been the subject of research aimed at understanding the genetic mechanisms underlying its resistance phenotype. In addition to being able to survive high doses of acute gamma radiation, the organism can grow under chronic gamma radiation and recover from a range of other DNA damaging conditions including exposure to desiccation, ultraviolet (UV) radiation and chemical genotoxic agents. This remarkable range of resistance has been attributed to efficient DNA repair processes capable of mending hundreds of single- and double-stranded DNA breaks without lethality or induced mutation. These characteristics were the impetus for sequencing the *D. radiodurans* genome, ongoing development for bioremediation, proteome and whole genome expression analyses. This thesis examines the relationships between radiation resistance, desiccation resistance, and thermotolerance in three novel *D. radiodurans* mutants (DR0070, DR0105, and DR2339). Mutants were tested for their ability to survive gamma radiation, desiccation, and elevated temperature. Results support that i) radiation resistance mechanisms responsible for survival following exposure to acute radiation are distinct from those needed for survival under chronic radiation, and ii) radiation resistance mechanisms responsible for survival under chronic radiation appear to be related to those involved with growth at elevated temperatures and desiccation resistance.

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List of Acronyms

Prefixes to the Names of Units

Mega (M)	10^6
Kilo (k)	10^3
Centi (c)	10^{-2}
Milli (m)	10^{-3}
Micro (μ)	10^{-6}

Units of Time

Hour	h
Minute	min
Second	sec

Units of Volume

Liter	l
Milliliter	ml
Microliter	μl

Units of Mass

Gram	g
Microgram	μg
Dalton	Da

Units of Concentration

Molar (mol/liter)	M
Millimolar	mM
Micromolar	μM

Units of Temperature

Degree Celsius	ºC

Units of Electricity

Ampere	A
Volt	V

Units of Radioactivity

Gray (Joule/Kg)	Gy
Kilo Gray	kGy

Physical and Chemical Quantities

Optical Density	OD

Miscellaneous Units

Hydrogen ion concentration, negative log of	pH

Other Standard Abbreviations and Symbols

About	~
Adenosine 5'-triphosphate	ATP
And	&
And Others	*et al.*
Base Pairs	bp
Colony Forming Unit	CFU
Deoxyribo Nucleic Acid	DNA
Deoxyribonuclease	DNase
United States Department of Energy	DOE
Double Strand Breaks	DSBs
Ethylenediaminetetraacetic Acid	EDTA
Gamma	γ
Greater than	>
Open Reading Frame	ORF
Percent	%
Polymerase Chain Reaction	PCR
Reactive Oxygen Species	ROS

RiboNucleic Acid	RNA
Ribosomal RNA/DNA	rDNA/rRNA
Sodium Dodecyl Sulphate	SDS
Ultraviolet	UV
Volume Per Volume	v/v
Weight Per Volume	w/v

Chapter 1: General Introduction

1.1. Description of *Deinococcus radiodurans*

1.1.1. General Characteristics

Bacteria belonging to the family *Deinococcaceae* are some of the most radiation-resistant organisms yet discovered. *D. radiodurans* strain R1 (ATCC BAA-816) was isolated originally in 1956 by A. W. Anderson and coworkers from canned meat that had spoiled after high exposure to gamma-rays (Anderson A. W., *et al*., 1956). *D. radiodurans* is a nonpathogenic, Gram-positive, nonsporulating, nonmotile, spherical, obligate aerobe (Murray R. G. E., 1986; Murray R. G. E., 1992). The 1 to 2 μm red-pigmented bacterium typically grows as clusters of four cells (tetracocci) in rich media (Thornley M. J., *et al*., 1965; Murray R. G. E., *et al.*, 1983; Work E., Griffith H., 1968; Brooks B. W., *et al*., 1980; Embley T. M., *et al*., 1987). Additionally, *D. radiodurans* is resistant to other DNA damaging conditions including exposure to desiccation, UV light, hydrogen peroxide, and numerous DNA damaging chemical agents (Moseley B. E., Evans D. M., 1983; Minton K. W., 1994; Wang P., Schellhorn H. E., 1995).

1.1.2. Phylogeny and Habitat

Members of the family *Deinococcaceae* were originally classified within the genus *Micrococcus* (Anderson A. W., *et al*, 1956). However, further taxonomic studies revealed that this classification was false, and construction of a phylogenic tree using deinococcal 16S rRNA gene sequences supported a separate genus '*Deinococcus*', with members that are closely related to *Thermus* sp. (Hensel R., *et al*., 1986; Weisburg W. G., *et al*., 1989; Rainey F. A., *et al*., 1997). Currently, the natural habitats of the deinococcal family are poorly defined since they have been isolated from diverse environments including soils (Brooks B. W., Murray R. G. E., 1981; Murray R. G. E., 1986;. Murray R. G. E., 1992), animal feeds (Ito H., *et al*., 1983), irradiated meat (Anderson A. W., *et al*, 1956; Davis N. S., *et al*.,1963; Maxcy R. B., Rowley D. B., 1978; Grant I. R., Patterson M. F., 1989), hot springs (Ferreira A. C., *et al*., 1997.), air, and textiles (Kristensen H., Christensen E. A., 1981; Christensen E. A., Kristensen H., 1981). A feature shared by

several of these environments is scarcity of water. The ability of *D. radiodurans* and the other deinococcal species to survive very prolonged periods of desiccation has given rise to the hypothesis that the extreme resistance phenotypes of *D. radiodurans* are secondary characteristics that are derived from its ability to survive DNA damage caused by cycles of desiccation and rehydration. Desiccation followed by rehydration causes double-stranded DNA breaks (DSBs), single-stranded DNA breaks (SSBs), and a wide range of nucleotide base damage (Mattimore V., Battista J. R., 1996) (See section 1.2. for more details).

1.1.3. The Genetics of *D. radiodurans*

The ability of *D. radiodurans* to survive very high doses of ionizing irradiation and its potential use for bioremediation of radioactive waste sites (Lange C. C., *et al*., 1998) was the impetus for the whole-scale genomic sequencing of this extremophile (White O., *et al*., 1999; http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gdr). The GC rich (66.6%) genome of *D. radiodurans* strain R1 (ATCC BAA-816) consists of two chromosomes (DR_Main [2.65 Mbp] and DR412 [412 kbp]), one megaplasmid (DR177 [177 kbp]), and one plasmid [46 kbp], encoding a total of 3,195 predicted genes (Makarova K. S., *et al*., 2001). In stationary-phase cultures of *D. radiodurans*, each cell contains four haploid copies of its genome, providing ample substrate for homologous recombination dependent processes (Daly M. J., Minton K. W., 1995 a). Cells which are in exponential growth phase contain 8-10 haploid genomic copies per cell (Hansen M. T., 1978; Harsojo, *et al*., 1981). A comprehensive genomic analysis (Makarova K. S., *et al*., 2001) revealed not only that the deinococcal genome contains about 1,000 genes of unknown function (uncharacterized genes), but also the presence of many typical bacterial genes, including those encoding DNA replication and repair. Remarkably, the number of known DNA repair genes encoded in *D. radiodurans* is less than other relatively radiation sensitive prokaryotes, such as *Escherichia coli* and *Bacillus subtilis* (Makarova K. S., *et al*., 2001). Several genes found in *D. radiodurans* have been detected previously only in eukaryotes and/or archaea, and the most likely explanation for this is that the shared genes were acquired by horizontal gene transfer (Makarova K. S., *et al*,

2001). Horizontal gene transfer is believed to be an extremely important contributor in the evolution of archaea and bacteria (Nelson K. E., *et al*., 1999). *D. radiodurans* is highly transformable with exogenously provided DNA, and it is possible that its propensity to acquire DNA played an important role in its evolution.

1.1.4. Transformability and Recombination of *Deinococcus*

Currently, *Deinococcus geothermalis* and *D. radiodurans* are the only deinococcal species for which genetic transformation and manipulation systems have been developed (Minton K. W., 1996; Brim H., *et al*., 2003). *D. radiodurans* has one of the highest reported levels of natural transformability, with DNA-marker-specific efficiencies as high as 3% when transformed in liquid culture (Daly M. J., *et al*., 1994 b; Minton K. W., 1994). High transformation frequencies facilitated the development of a variety of techniques for genetic manipulation of this organism (Daly M. J., *et al*., 1994 a; Daly M. J., Minton K. W., 1997; Lange C. C., *et al*., 1998; Brim H., *et al*., 2000). Deinococcal expression systems are based on genomic integration vectors as well as autonomous plasmids, both of which have been used to disrupt genes or complement mutants (Lange C. C., *et al*., 1998; Brim H., *et al*., 2000). Typically for disruptive genomic integration, mutants are generated using *E. coli*-based plasmids that target homologous deinococcal genome sequences. Integration into the target sequences is forced by selection with antibiotics encoded within the transforming constructs. For *Deinococcus* gene disruptions, the vectors encode kanamycin, chloramphenicol, and/or tetracycline resistance ($Km^R/Cm^R/Tc^R$) plus an internal (~250-700 bp) fragment of a *D. radiodurans* gene (*e.g*., generated by PCR). Such an insertion vector is able to integrate into a recipient's genome by a single crossover (by tandem duplication) at the site of homology (Brim H., *et al*., 2000). If the entire integrating sequence of a transforming vector lies within the boundaries of a gene, the targeted gene will be disrupted. Several rounds of antibiotic selection following transformation can yield homozygosity for non-essential genes. However, permanent heterozygosity in the presence of strong antibiotic selection does occur, and indicates that some copies of the gene are disrupted while others are not, and that the gene is essential (Matrosova V. Y., *et al*., 2003). Homozygosity/heterozygosity can be determined by detailed mapping of the integration

site by Southern blot analysis using diagnostic radiolabeled probes. The procedure of duplication insertion is not restricted to deactivation of one gene, but can be used to disrupt another gene in the same cell if the second targeted gene is tagged by a different antibiotic resistance marker. For example, double mutants can be generated by disrupting one gene with a Km^R-encoding disruption vector while another gene in the same cell can be disrupted with a vector encoding Cm^R.

1.1.5. The Extreme Resistance of *D. radiodurans* to DNA Damage

An organism's ability to survive exposure to radiation is typically assayed by measuring cell survival on nutrient agar plates using appropriate dilutions of irradiated cultures. The dose yielding 37% colony-forming unit (CFU) survival is a metric standard of resistance that allows comparison with other organisms being investigated for radiation resistance. This dose is referred to as the D_{37} value. D_{10} values, that correspond to 10% CFU survival are also commonly used. The ionizing radiation specific D_{37} value of *D. radiodurans* growing logarithmically in rich medium is about 16 times higher than the D_{37} value of similarly cultured *E. coli* (Gaidamakova E. K., *et al*., 2003). In stationary-phase, the relative resistance of *D. radiodurans* to ionizing radiation is even greater (Daly M. J., *et al*., 1994 b; Minton K. W., 1994). DSBs are considered to be the most lethal form of DNA damage because they destroy the linear integrity of DNA where genetic information is lost at the break site, and where single-stranded DNA is unavailable for template-dependent repair (Kuzminov A., 1999). Whereas *E. coli* is capable of repairing only a small number of DSBs per chromosome (Krasin F., Hutchinson F., 1977), *D. radiodurans* can repair >100 DSBs per chromosome within a few hours (Kitayama S., Matsuyama A., 1971; Daly M. J., *et al*., 1994 b; Lin J., *et al*., 1999). During exponential growth phase, *E. coli* maintains four to five copies of its genome, which have been shown to be necessary for DSB repair (Krasin F., Hutchinson F., 1977). However, multiple genome copies in *D. radiodurans* are not sufficient to explain its remarkable resistance. For example, *Azotobacter vinelandii*, *Micrococcus luteus*, and *Micrococcus sodonensis* all contain multiple genome equivalents but are relatively radiation sensitive (Moseley B. E., Evans D. M., 1983; Majumdar S., Chandra A. K., 1985; Punita S. J., *et al*., 1989), and experiments in which the number of genome

copies was altered in *D. radiodurans* revealed little correlation with its resistance profile (Harsojo, *et al*., 1981). Although considerable progress in understanding *D. radiodurans* resistance mechanisms has been made over the last decade, the genetic repair systems still remain mostly undefined. For sure, *recA*-dependent recombination mechanisms are very important to deinococcal recovery (Daly M. J., Minton K. W., 1996), but given the similarity of its RecA to other bacteria, much remains unknown. The lack of a clearly identifiable unique DNA repair system in *D. radiodurans* has given rise to three competing views of the mechanisms responsible for its extraordinary survival (Battista J. R., *et al*., 1999); i) there are novel repair functions encoded among hypothetical genes predicted by genomic annotation (White O., *et al*., 1999; Makarova K. S., *et al*., 2001; Liu Y., *et al*., 2003); or ii) *D. radiodurans* uses conventional DNA repair pathways, but with much greater efficiency than other bacteria (Sweet D. M., Moseley B. E., 1974; Daly M. J., Minton K. W., 1996; Battista J. R., *et al*., 1999; White O., *et al*., 1999; Makarova K. S., *et al*., 2001; Liu Y, *et al*., 2003); or iii) DNA repair in *D. radiodurans* is facilitated by its ringlike chromosomal structures (Daly M. J., Minton K. W., 1995 b; Levin-Zaidman S., *et al*., 2003).

1.2. Evolution of the Radiation Resistance Phenotype

Of the seven reported species that make up the family *Deinococcaceae*, *D. radiodurans* is the most characterized, and the consensus opinion within the deinococcal community is that the remarkable resistance of these organisms is a result of unusually efficient DNA repair (Moseley B. E. B., 1983; Smith M. D., *et al*., 1992; Minton K. W., 1994). The average terrestrial annual exposure to natural radiation sources (Cosmic and terrestrial sources) is estimated to be 0.0005-0.0024 Gy/year (United Nations Scientific Committee on the Effects of Atomic Radiation, 1982; United Nations Scientific Committee on the Effects of Atomic Radiation, 2000). Background radiation levels, therefore, are relatively low compared to the dose rates at which *Deinococcus* bacteria can grow (60 Gy/hour) (Venkateswaran A., *et al*., 2000). There are no known natural terrestrial environments which produce levels of ionizing radiation sufficient to explain the evolution of organisms like *D. radiodurans* (Makarova K. S., *et al*., 2001). Exposure to ionizing radiation induces DSBs, which are also induced at high levels by cycles of

dehydration/rehydration (Mattimore V., Battista J. R., 1996). Mechanisms to protect against the damaging effects of desiccation have evolved in numerous phylogenetically diverse organisms (Crowe J. H., *et al*., 1997). For example, *Enterococcus* sp., *Lactobacillus* sp., and *Arthrobacter* sp. are all highly desiccation resistant (Boylen C. W., 1973; Bale M. J., *et al*., 1993; Linders L. J., *et al*., 1997). Such organisms are also known for their resistance to ionizing radiation, but systematic comparisons of their recovery capabilities are lacking. Previous work reported on *D. radiodurans* has shown that radiation sensitive mutants are also desiccation sensitive, suggesting that there is a strong correlation between these two phenotypes (Mattimore V., Battista J. R., 1996).

1.3. Thesis Objectives

Annotation of the complete *D. radiodurans* genome sequence has so far failed to identify DNA repair pathways unique to this organism (White O., *et al*., 1999; Makarova K. S., *et al*., 2001). Furthermore, experimental efforts based on whole genome transcriptome analyses for *D. radiodurans* recovering from irradiation have also revealed little new information regarding the repertoire of DNA repair genes involved in recovery (Gutman P. D., *et al*., 1994; Makarova K. S., *et al*., 2001; Earl A. M., *et al*., 2002 b; Liu Y, *et al*., 2003; Hua Y., *et al*., 2003). This thesis builds on recent genomic and experimental data generated for *D. radiodurans* and addresses the possibility that novel genes may function alone or in combination during cellular recovery from DNA damaging conditions (*i.e*., growth at elevated temperatures, desiccation, and resistance to γ radiation).

1. **Characterization of previously constructed single mutants containing disruptions in genes implicated in recovery.** Three *D. radiodurans* mutants previously constructed based on prioritization of genes identified by a combination of genomic informatic and experimental results were characterized for their effect on growth at different temperatures, desiccation resistance, and resistance to chronically and acutely delivered γ radiation. A published experimental assay to measure desiccation resistance was improved.

2. **Construction and characterization of double mutants containing disruptions in two different genes implicated in recovery.** Seven distinct double *D. radiodurans* mutants were constructed by transformation using high molecular weight DNA prepared from the single mutants reported in Chapter 2, followed by double antibiotic selection. Numerous isolates from each of the double mutants were evaluated for their resistance to both chronic and acute radiation. Additional double mutants were constructed for future evaluation.

3. **Genetic characterization of a double mutant displaying increased sensitivity to radiation compared to either of its single mutant parental strains**. *D. radiodurans* encodes two distinct DNA ligases (DR2069 and DRB0100). Single mutants of each of these ligases show wild-type levels of resistance. However, the double mutant construct ($DRB0100^{-}Cm^{R}$ + $DR2069^{-}Km^{R}$) was shown to be sensitive to acute γ radiation. Detailed genomic mapping by Southern blot analysis was used to characterize the mutant strain.

Chapter 2: Characterization of Previously Constructed Single *D. radiodurans* Mutants Containing Disruptions in Genes Implicated in Recovery

2.1. Introduction

Results from the annotation of the whole *D. radiodurans* genome sequence (Makarova K. S., *et al*., 2001) and from DNA-microarray expression analysis of cells undergoing recovery following exposure to ionizing radiation (Liu Y., *et al*., 2003) were used to help identify genes that encode the extreme resistance phenotype. Several genes identified are known to be involved in DNA replication, repair, and/or recombination in other organisms. Of the uncharacterized group of genes shown to have elevated expression levels after irradiation, several were selected for further investigation by targeted gene disruption. Table 2.1 lists the uncharacterized genes examined in this chapter. For example, the uncharacterized gene DR0070 was disrupted by tandem duplication insertion, mapped by Southern blotting, and shown to be relatively radiation sensitive compared to the wild-type (Liu Y., *et al*., 2003). Phenotype studies for single mutants presented in this chapter focused on i) γ radiation resistance; ii) resistance of growth to elevated temperatures, known to cause DNA damage in *E. coli* (Michel B., *et al*., 1997; Hanada K., *et al*., 2001); and iii) desiccation resistance (Mattimore V., Battista J. R., 1996).

Unlike studies on the effects of irradiation and desiccation in *D. radiodurans*, until recently there has been no systematic research on the effects of different temperatures on growth and survival of *D. radiodurans* resistance mutants (Harada K., *et al*., 2003). The optimum growth temperature of wild-type *D. radiodurans* is known to be about 32˚C, and the highest temperature able to sustain growth being ~39˚C (Bruce A. K., 1964; Earl A. M., *et al*., 2002 a; Lipton M. S., *et al*., 2002). Mutant growth at 37˚C was examined. The ability of deinococcal cells to grow at 37˚C may be an indicator of its normal DNA replication and repair capabilities. This possibility was examined by testing one mutant ($DR2339^{-}Km^{R}$) for its ability to grow at 37˚C; DR2339 displays diminished growth characteristics under chronic radiation.

Table 2.1. Single mutants

Name of gene disruption/ strain[a]	Length of gene targeted for disruption [bp][b]	Hypothetical function of uncharacterized protein[c]	Expression pattern [fold-increase][d]	Genotype of disrupted gene[e]	Resistance to acute/chronic radiation[f]
DR0070$^-$Km^R	600	alkaline protease,	3	homozygous	sensitive/resistant
DR0105$^-$Km^R	412	desiccation resistance protein of the LEA76 family	12	homozygous	resistant/resistant
DR2339$^-$Km^R	705	2'-5'RNA ligase (LigT)	14	homozygous	resistant/sensitive
DR2069$^-$Km^R	2,103	NAD$^+$ dependant DNA ligase	1	heterozygous	resistant/resistant
DR0140$^-$Km^R	477	no hypothetical function	6	homozygous	resistant/resistant
DR1916$^-$Km^R	2,255	RecG helicase	3	homozygous	resistant/resistant
DR2482$^-$Km^R	1,479	similar to sigma factor	6	homozygous	resistant/resistant
DRB0100$^-$Km^R	633	protein homologue to eukaryotic DNA ligase III	14	homozygous	resistant/resistant
DRC0012$^-$Km^R	540	transcriptional regulator	7	homozygous	resistant/resistant
DR0070$^-$Cm^R	600	alkaline protease	3	homozygous	sensitive/resistant
DR2482$^-$Cm^R	1,479	similar to sigma factor	6	homozygous	resistant/resistant
DRB0100$^-$Cm^R	633	protein homologue to eukaryotic DNA ligase III	14	homozygous	resistant/resistant

Footnotes Table 2.1

[a]Strain name is based on the gene ID of the disrupted gene (Makarova K. S., *et al*., 2001) and on the antibiotic resistance encoded in the disruption vector. Kanamycin resistance (Km^R) was obtained by pCR2.1(Invitrogen, CA) mediated gene disruption. Chloramphenicol resistance was obtained by pPCR-Script Cam SK(+) (Stratagene, CA) mediated gene disruption. If not mentioned otherwise the mutants are kanamycin resistant.

[b]Gene size of predicted hypothetical protein (Makarova K. S., *et al*., 2001).

[c]Gene function of predicted hypothetical protein (Makarova K. S., *et al*., 2001).

[d]Expression pattern of the wild-type genes during recovery after exposure to acute radiation (15 kGy) (Liu Y, *et al*., 2003). (fold-increase) relative to the non-irradiated control.

[e]Genotype of the disrupted gene was determined by Southern analysis using ^{32}P radiolabeled probes (Gaidamakova E. K., *et al*., 2003).

[f]Resistance to radiation was determined after exposure to acute (^{60}Co: 9 kGy) and chronic (^{137}Cs: 50 Gy/h) radiation.

Life under conditions where water is restricted ("anhydrobiosis") requires maintenance of DNA and protein structure and membrane integrity, as well as a myriad of mechanisms needed to minimize damage caused by oxidative stress, which occurs during recovery from desiccation. Consistently, several organisms able to resist desiccation are also known for their ability to resist radiation and heat-shock (Christensen E. A., 1964; Crowe L. M., Crowe J. H., 1992; Bale M. J., *et al*., 1993). Desiccation survival mechanisms have evolved in phylogenetically diverse organisms, including bacteria, crustacea, higher and lower plants, yeast cells, and multicellular animals like the tardigrade (Crowe J. H., *et al*., 1997; Jonsson K. I., Rebecchi L., 2002). These organisms typically enter a stage of dormancy when water is restricted. Many bacteria have developed additional defenses including the formation of spores which have very low water contents and which show no metabolic activity (Adams D. G., 2000; Nicholson W. L., *et al*., 2000). *D. radiodurans* is one of only a few bacteria reported that has evolved to survive dehydration in a vegetative state (Mattimore V., Battista J. R., 1996; Battista J. R., *et al*., 2001; Saffary R., *et al*., 2002). In 1996, Mattimore and Battista showed that dehydration induces DSBs in *D. radiodurans* and that the extent of DNA damage was comparable to that caused by high dose γ-radiation. Using the desiccation resistance protocol developed by Battista, mutants investigated in this chapter were tested for their resistance to desiccation over a period of weeks. The desiccation protocol was further developed to more accurately record the survival capabilities of the strains under investigation. The aims of this chapter follow:

1. To test the possibility that a mutant's growth characteristics at elevated temperatures are related to its ability to survive radiation.

2. To test the relationship between radiation and desiccation resistance for the mutants under investigation.

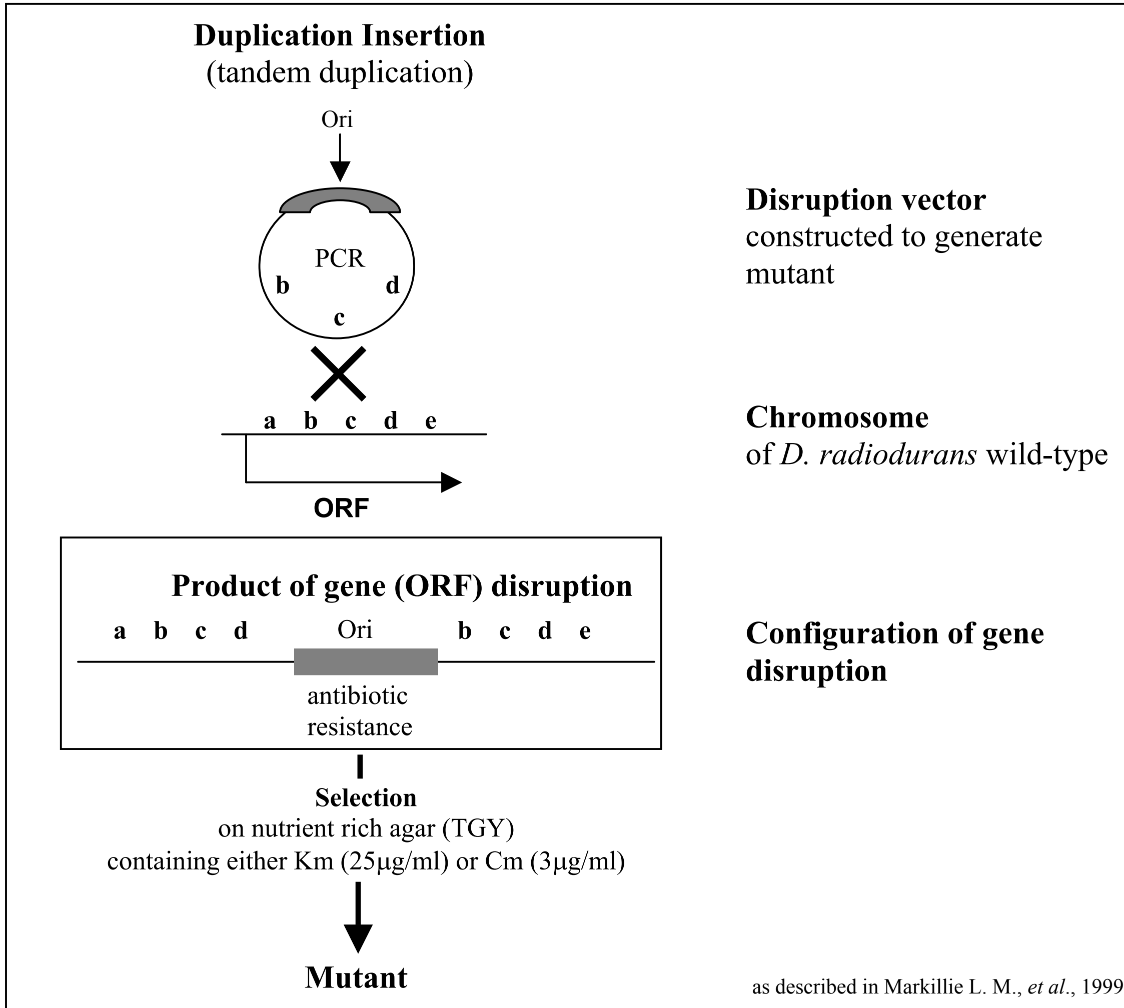
3. To test the hypothesis that ORF DR0105, which shows homology to a plant desiccation resistance gene, contributes to desiccation resistance in *D. radiodurans*.

2.2. Material and Methods

2.2.1. Generation of Single Mutants

Background: Results from the annotation of *D. radiodurans* (Makarova K. S., *et al*., 2001) and from analysis of transcriptome dynamics following acute irradiation (Liu Y., *et al*., 2003) were used to identify predicted genes that might play a role in the extreme resistance phenotype. To date, forty selected genes have been inactivated by duplication insertion (Fig. 2.1) (Gaidamakova, E. K. *et al*., 2003). Typically, a ~4 kb autonomously replicating *E. coli* plasmid [pCR2.1 encoding kanamycin resistance (Km^R) or pPCR-Script Cam SK(+) encoding chloramphenicol resistance (Cm^R)] that contains a fragment of *D. radiodurans* genomic DNA is used to knock out genes (ORFs) (Markillie L. M., *et al*., 1999) (Fig. 2.1). Disruption vectors for the selected genes were generated by cloning DNA fragments (250-700 bp) obtained by PCR amplification into position 295 of the *E. coli* plasmid pCR2.1 (Invitrogen, CA) or into position 728 of the *E. coli* plasmid pPCR-Script Cam SK(+) (Stratagene, CA) according to the manufacturer's protocols. Purified disruption vectors were then transformed into *D. radiodurans* with kanamycin (Km) selection (25 μg/ml; USB, OH) or chloramphenicol (Cm) selection (3 μg/ml; USB, OH) as described previously (Daly M. J., *et al*., 1994 b) and as summarized in Fig. 2.1. To determine homozygosity or heterozygosity of an ORF disruption, total DNA preparations from the wild-type strain and from a transformant strain were subjected to restriction endonuclease mapping and Southern blotting with diagnostic ^{32}P radiolabeled probes as described in Liu Y., *et al*., 2003. This approach generates diagnostic maps of a transformant's integration site. If heterozygosity for a disruption of a gene is confirmed after several rounds of antibiotic selection, the target gene is assumed to be essential (Matrosova V. Y., *et al*., 2003). Cell viabilities of single homozygous mutants were determined after exposure to chronic radiation at 50 Gy/hour (^{137}Cs Gammacell 40 irradiation unit [Atomic Energy of Canada Limited]) and after high level acute γ-radiation (5-20 kGy) (8 kGy/hour ^{60}Co, Gammacell irradiation unit [J. L. Shepard and Associates, Model 109]) as described elsewhere (Gaidamakova E. K., *et al*., 2003).

Figure 2.1. Generation of *D. radiodurans* mutants by duplication insertion



Legend Fig. 2.1. The segment **bcd** of the disruption vector is a 250-700 bp internal sequence of a *D. radiodurans* gene targeted for disruption. The thick gray segment represents *E. coli* sequences (pPCR and pCR based plasmids [Stratagene, CA and Invitrogen, CA]) including an *E. coli* origin of replication (Ori) and an antibiotic resistance gene (Km^R or Cm^R) The plasmid will integrate into the recipient's genome by a single crossover at the site of homology. Transformants are subjected to several rounds of selection on TGY containing antibiotics.

The box surrounding the product of gene disruption represents the disrupted gene and its flanking sequence.

For investigations described in this chapter, selected single mutants (Table 2.1) were further characterized for growth and desiccation resistance.

2.2.2. Phenotypic Analysis of Single Mutants

2.2.2.1. Bacterial Strains

The bacterial strains used in this study are listed in Table 2.1. *D. radiodurans* strains were pre-grown at 32˚C in TGY broth [1% bactotryptone (Difco, NJ), 0.5% yeast extract (Sigma, MO), and 0.1% glucose (Sigma, MO)] or on TGY agar plates solidified with 1.5% Bacto-agar (BD, NJ), containing appropriate antibiotics as indicated in Table 2.1 and 2.2. Liquid cultures were grown at 32°C with aeration.

2.2.2.2. Quantifying Growth

Single colonies of strains DR0070, DR0105, DR2339 and control strain (Table 2.2) were transferred from TGY plates to 5 ml TGY broth containing the appropriate antibiotic, using sterile technique. Cells were grown at 32˚C with aeration to OD_{600} ~1 (OD_{600} = 1.0 ~ 1 x 10^{8} CFUs/ml [Daly M. J., *et al*., 1994 a]). Optical density was determined using a Beckman spectrophotometer. One ml of each sample was transferred to 14 ml TGY containing the appropriate antibiotic (Table 2.2). Samples were incubated at 32˚C, 37˚C, or 42˚C. 100 µl of each sample were diluted in 900 µl TGY for measuring OD_{600} at the indicated time points (Table 2.2). The time taken to reach maximum cell density/OD_{600} was determined by plotting OD_{600} against incubation time. Each data set presented was derived from three independent trials with standard deviations shown (*e.g*., Fig. 2.2).

Table 2.2. Bacterial strains for which growth dynamics at different temperatures were examined

***D. radiodurans* strain**	**Concentration of antibiotic in media**	**Time [hours] at which optical density was measured**
R1	-	0, 2.2, 5.6, 8.8, 24.8, 53.1, 141.2
DR0070	25 µg/ml kanamycin	0, 2.2, 5.6, 8.8, 24.8, 53.1, 141.2
DR0105	25 µg/ml kanamycin	0, 2.2, 5.6, 8.8, 24.8, 53.1, 141.2
DR2339	25 µg/ml kanamycin	0, 2.2, 5.6, 8.8, 24.8, 53.1, 141.2

2.2.2.3. Desiccation Protocols

Cells were pre-grown in TGY to OD_{600} ~1.0, isolated by centrifugation (1,331 x *g*, 5 min, 4˚C) and then re-suspended at OD_{600} = 0.25 in either 10 mM $MgSO_4$ (Sigma, MO) or fresh TGY. Viable cell counts (CFU values) for the cultures were determined prior to the desiccation experiments by plating appropriate dilutions (10^0 to 10^{-7}) on TGY. For desiccation resistance assays, ~1 x 10^6 cells were transferred to a glass slide and placed in a desiccation chamber over anhydrous calcium sulfate (WA Hammond Drierite CO. LTD., OH) containing a visible indicator. The desiccation chamber was hermetically sealed and stored at room temperature. At intervals of one week, extending to 6 weeks, glass slides were removed, cells were re-suspended in 1 ml TGY or 500 µl of 10 mM $MgSO_4$, and CFU survival values were determined by plating appropriate dilutions (10^1 to 10^{-7}) of cells onto TGY plates. Survival frequencies were determined by comparing CFU values from desiccated samples to the viable cell counts of the original cultures, after cells had been allowed to recover for five days. Each data set presented was derived from three independent trials with standard deviations shown (*e.g*., Fig. 2.6).

2.3. Results

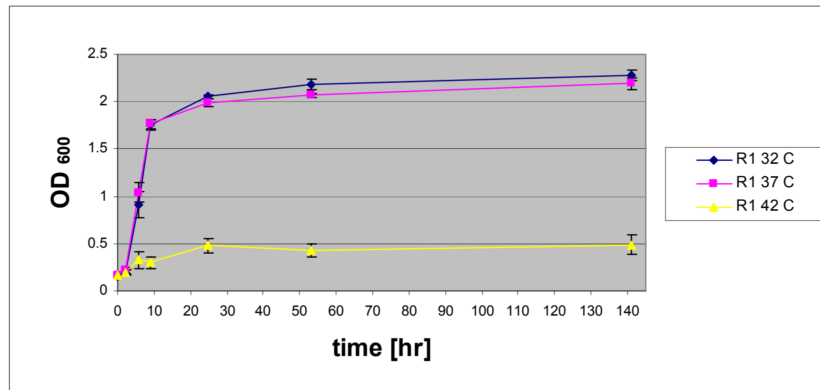
2.3.1. Background: Mutant Construction and Radiation Resistance Phenotypes

From 40 ORFs disrupted by duplication insertion (Fig. 2.1), only two showed diminished resistance to ionizing radiation (Table 2.1). The most sensitive mutant was DR0070 that is sensitive to acute radiation, but not chronic radiation. In contrast, mutant DR2339 was resistant to acute radiation, but sensitive to chronic radiation. Attempts to generate homozygous mutants for DR2069 were not successful, indicating that this gene is essential. Table 2.1 summarizes the results of mutant analysis for radiation resistance, annotation (Makarova K. S., *et al.,* 2001), and expression analysis (15 kGy) (Liu Y., *et al*., 2003).

2.3.2. Identification of Mutants Sensitive to Elevated Temperature

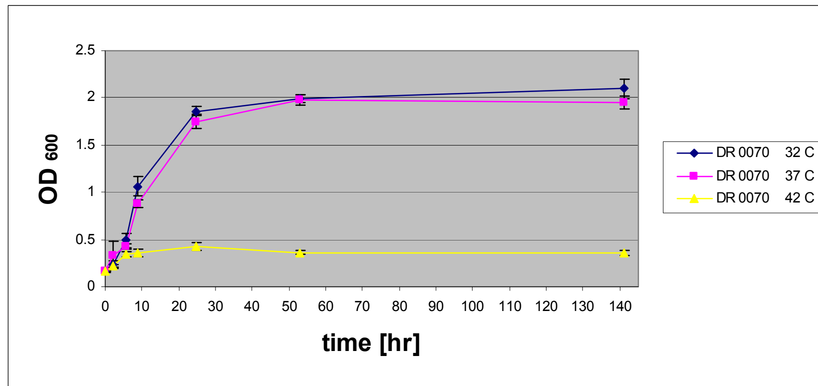
Strains R1, DR0070, DR0105, and DR2339 were evaluated for their ability to grow at the elevated temperature of 37°C (Figs. 2.2, 2.3, 2.4, & 2.5, respectively). All strains grew at 32°C with growth kinetics essentially the same as wild-type. As expected, none of the strains were able to grow at 42°C. However, at 37°C strain DR2339 (2'-5' RNA ligase) showed significantly diminished growth compared to the other strains. Whereas R1, DR0070, and DR0105 entered exponential growth phase at 10 hours and stationary phase at 30 hours, mutant DR2339 did not display a typical S-shaped growth curve. Rather, it displayed a linear-type growth curve and did not reach wild-type stationary-phase cell density levels (30 h) until 140 hours after inoculation.

Figure 2.2. Growth of *D. radiodurans* R1 at 32°C, 37°C, and 42°C



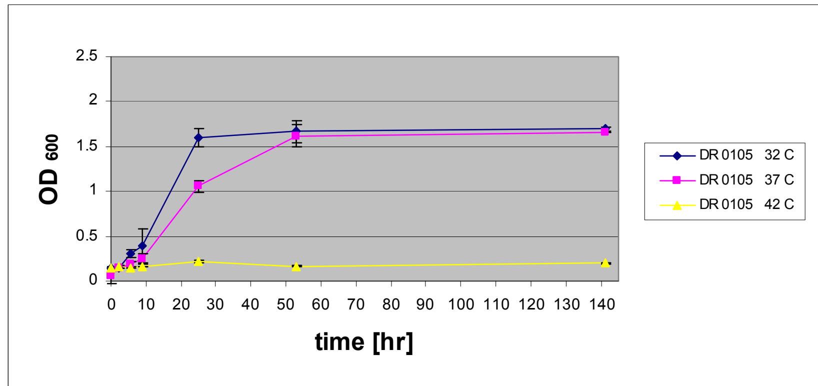
Legend Fig. 2.2. Cell density was determined by measuring the optical density of cultures at 600 nm. Cells were cultured in TGY. OD_{600} 1.0 ~ 1 x 10^{8} CFU/ml. Incubations were in triplicate and standard deviations are shown.

Figure 2.3. Growth of *D. radiodurans* DR0070 at 32°C, 37°C, and 42°C



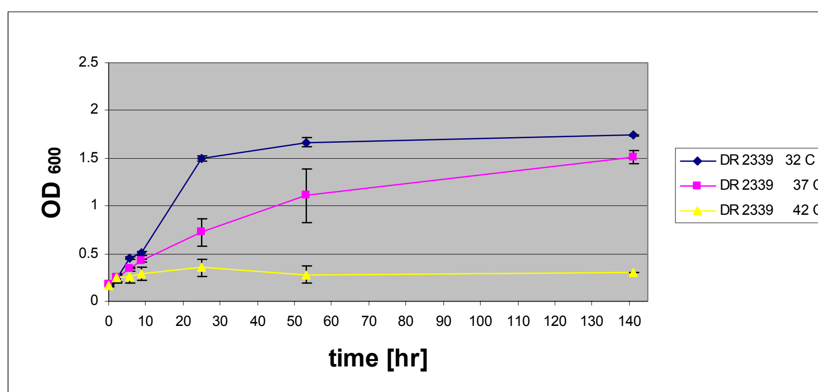
Legend Fig. 2.3. Cell density was determined by measuring the optical density of cultures at 600 nm. Cells were cultured in TGY containing 25 μg/ml kanamycin. OD_{600} 1.0 ~ 1 x 10^8 CFU/ml. Incubations were in triplicate and standard deviations are shown.

Figure 2.4. Growth of *D. radiodurans* DR0105 at 32°C, 37°C, and 42°C



Legend Fig. 2.4. Cell density was determined by measuring the optical density of cultures at 600 nm. Cells were cultured in TGY containing 25 μg/ml kanamycin. OD_{600} 1.0 ~ 1 x 10^8 CFU/ml. Incubations were in triplicate and standard deviations are shown.

Figure 2.5. Growth of *D. radiodurans* DR2339 at 32°C, 37°C, and 42°C



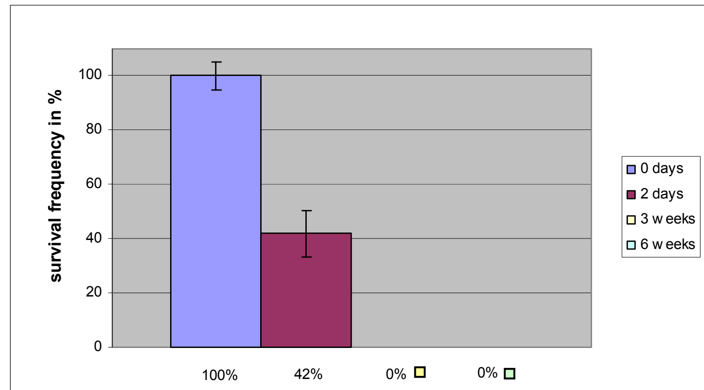
Legend Fig. 2.5. Cell density was determined by measuring the optical density of cultures at 600 nm. Cells were cultured in TGY containing 25 μg/ml kanamycin. OD_{600} 1.0 ~ 1 x 10^8 CFU/ml. Incubations were in triplicate and standard deviations are shown.

2.3.3. Resistance to Desiccation

Strains R1, DR0070, DR0105, and DR2339 were evaluated for desiccation resistance using two distinct protocols. The first protocol evaluated desiccation resistance of cells prepared in 10 mM $MgSO_4$. This method was previously used by Mattimore and Battista (1996) to evaluate desiccation recovery capabilities, but was found to be unreliable based on the following observation. The expected desiccation survival of *D. radiodurans* R1 prepared in 10 mM $MgSO_4$ after 2 weeks of desiccation was expected to be ~100% (Mattimore V. and Battista J. R., 1996). However, the results of using this $MgSO_4$-based protocol could not be replicated. Instead, we found that treatment of any *Deinococcus* cells with 10 mM $MgSO_4$ rendered them desiccation sensitive (Fig. 2.6). All $MgSO_4$-treated wild-type cells were killed by desiccation after 3 weeks and a short period of desiccation of only two days yielded only 42% survival (Fig. 2.6). Figure 2.7 and 2.8 show similar desiccation sensitivities for a *D. radiodurans* strain containing an autonomously replicating plasmid pMD66 (strain MD68), that encodes Km resistance, and for the mutant DR0105. Together, these results support that the $MgSO_4$-based assay

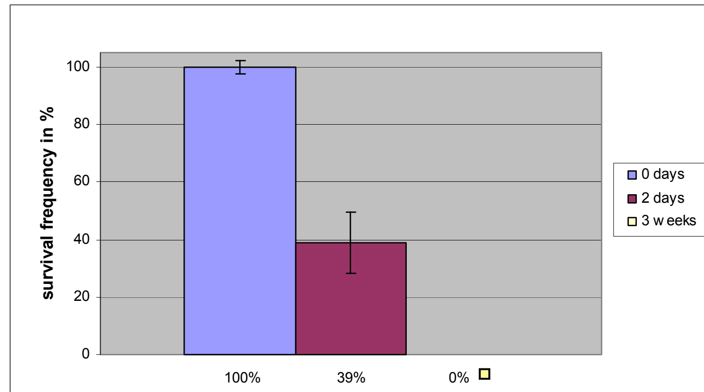
was not suitable to characterizing the desiccation resistance phenotype using this laboratories cultivation methods. Reasons for the contradiction between the findings reported here and those reported by Battista are unknown. As an alternative, the desiccation assay was successfully repeated using TGY as the substrate prior to transferring cells to the desiccation chamber.

Figure 2.6. Survival frequencies for wild-type *D. radiodurans* pre-grown in TGY and desiccated in 10 mM $MgSO_4$



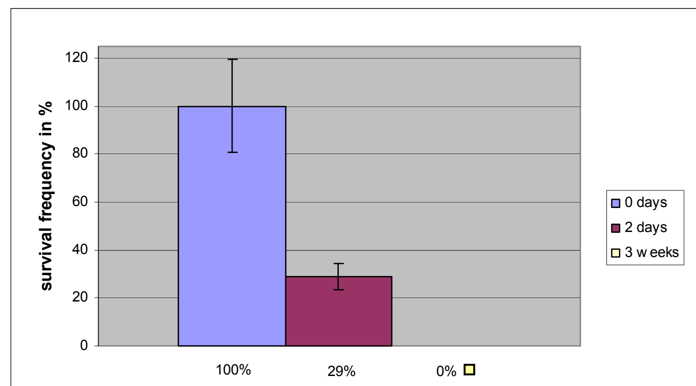
Legend Fig. 2.6. Cells were recovered and CFU values were determined in triplicate as described in section 2.2.2.3. Standard deviations are shown.

Figure 2.7. Survival frequencies for *D. radiodurans* MD68 pre-grown in TGY and desiccated in 10 mM $MgSO_4$



Legend Fig. 2.7. Cells were recovered and CFU values were determined in triplicate as described in section 2.2.2.3. Standard deviations are shown. Survival frequencies determined after recovery from six weeks of desiccation are not shown. MD68 is wild-type *D. radiodurans* containing the Km^R-encoding plasmid pMD66.

Figure 2.8. Survival frequencies for *D. radiodurans* DR0105 pre-grown in TGY and desiccated in 10 mM $MgSO_4$



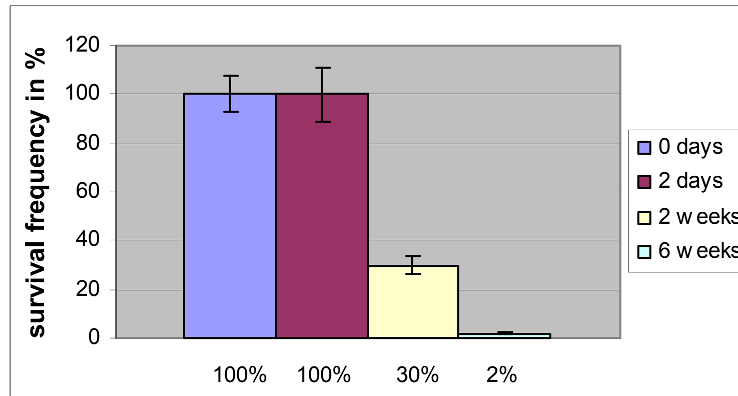
Legend Fig. 2.8. Cells were recovered and CFU values were determined in triplicate as described in section 2.2.2.3. Standard deviations are shown. Survival frequency determined after recovery from six weeks of desiccation are not shown.

The second desiccation approach utilizing TGY as an alternative to $MgSO_4$ confirmed that wild-type is very resistant to desiccation (Fig. 2.9). For example, 30% survival for wild-type was observed after 2 weeks of desiccation. Included in these investigations was a wild-type *D. radiodurans* strain containing the Km^R-encoding autonomously replicating plasmid pMD66 (strain MD68) as a control, since the single mutant strains under investigation (Table 2.3) were all grown with Km selection (Fig. 2.10). Analysis of the desiccation resistance characteristics showed that DR0070 was not more sensitive than MD68 (wild-type) (Fig. 2.11); that DR0105 was more resistant than MD68 (Fig. 2.12); and that DR2339 was more sensitive than MD68 (Fig. 2.13). In summary, these findings suggest that the gene DR0070 is involved with radiation resistance (Table 2.1), but not desiccation resistance (Fig. 2.11); that DR2339 is involved in radiation (Table 2.1) and desiccation resistance (Fig. 2.13); and that DR0105 is not involved in either radiation or desiccation resistance (Table 2.1 & Fig. 2.12).

Table 2.3. Bacterial strains pre-grown and desiccated in TGY

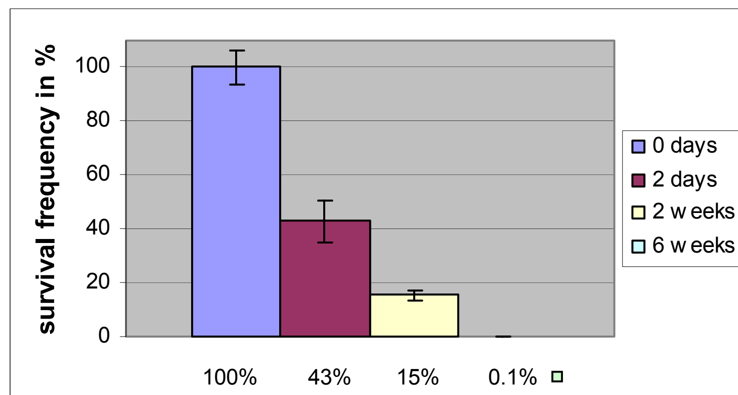
***D. radiodurans* strain**	**Concentration of antibiotic in media**	**Length of desiccation**
R1	-	2 days, 2 weeks, 6 weeks
MD68	25 μg/ml kanamycin	2 days, 2 weeks, 6 weeks
DR0070	25 μg/ml kanamycin	2 days, 2 weeks, 6 weeks
DR0105	25 μg/ml kanamycin	2 days, 2 weeks, 6 weeks
DR2339	25 μg/ml kanamycin	2 days, 2 weeks, 6 weeks

Figure 2.9. Survival frequencies for *D. radiodurans* R1 pre-grown and desiccated in TGY



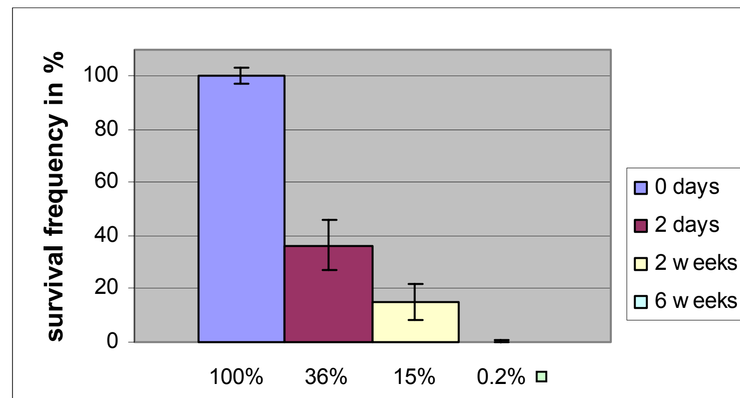
Legend Fig. 2.9. Cells were recovered and CFU values were determined in triplicate as described in section 2.2.2.3. Standard deviations are shown.

Figure 2.10. Survival frequencies for *D. radiodurans* MD68 pre-grown and desiccated in TGY



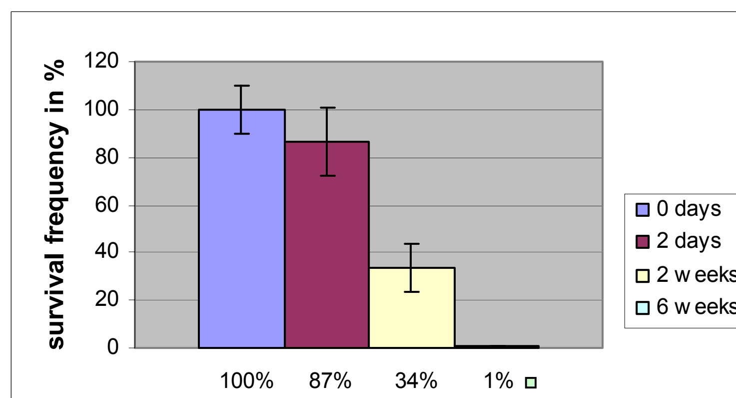
Legend Fig. 2.10. Cells were recovered and CFU values were determined in triplicate as described in section 2.2.2.3. Standard deviations are shown.

Figure 2.11. Survival frequencies for *D. radiodurans* DR0070 pre-grown and desiccated in TGY



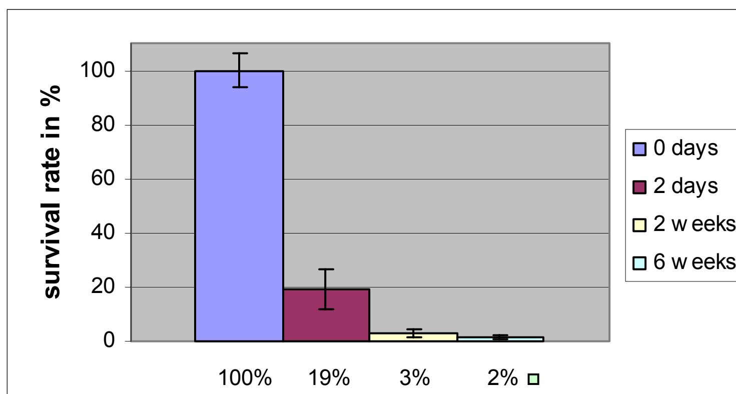
Legend Fig. 2.11. Cells were recovered and CFU values were determined in triplicate as described in section 2.2.2.3. Standard deviations are shown.

Figure 2.12. Survival frequencies for *D. radiodurans* DR0105 pre-grown and desiccated in TGY



Legend Fig. 2.12. Cells were recovered and CFU values were determined in triplicate as described in section 2.2.2.3. Standard deviations are shown.

Figure 2.13. Survival frequencies for *D. radiodurans* DR2339 pre-grown and desiccated in TGY



Legend Fig. 2.13. Cells were recovered and CFU values were determined in triplicate as described in section 2.2.2.3. Standard deviations are shown.

Table 2.4. Summary of results

Strain name[a]	**Hypothetical function of uncharacterized protein**[c]	**Resistance to acute /chronic radiation**[f] (Table 2.1)	**Resistance to desiccation** (Figs. 2.11, 2.12, and 2.13)	**Growth at 37°C** (Figs. 2.3, 2.4, and 2.5)
DR0070	Alkaline protease	sensitive/resistant	resistant	yes
DR0105	Desiccation resistance protein	resistant/resistant	resistant	yes
DR2339	2’-5’RNA ligase (LigT)	resistant/sensitive	sensitive	diminished

Footnotes in Table 2.4 are as in Table 2.1.

2.4. Discussion

The chronic and acute irradiation resistance phenotypes of three *D. radiodurans* mutants (Table 2.4) were compared to their ability to grow at the elevated temperature of 37°C and to resist desiccation. The three mutants selected for investigation were previously implicated by computational and experimental approaches as participating in the extreme radiation resistance phenotype of *D. radiodurans*.

DR0070 was originally identified by high-throughput proteomics (Lipton M. S., *et al*., 2002) as a protein expressed predominantly under alkaline conditions, and was tentatively assigned the name 'alkaline protease' based on comparative genomics. However, this functional assignment still needs to be confirmed. Expression of DR0070 has been reported to be induced 3-fold following an acute dose of 15 kGy (Table 2.1) and was selected for disruption by tandem duplication (Liu Y., *et al*., 2003). A homozygous disruption of DR0070 confirmed the gene as participating in recovery from acute radiation, but its identity as a DNA repair gene has not yet been established. Based on the sensitivity of DR0070 to acute radiation (for dormant cells at 0°C) but not to chronic radiation (for growing cells) (Table 2.1), some of the mechanisms involved in resistance to acute radiation may be distinct from those needed for survival under chronic radiation. This is an important distinction because previous assessments of genes believed to be involved in resistance to acute radiation were inferred to be involved in resistance to chronic radiation. For example, in contrast to DR0070, *recA* mutants of *D. radiodurans* are sensitive to acute radiation and unable to grow under chronic radiation (Daly M. J., 2000). These findings illustrate the complexity of the resistance phenotype of *D. radiodurans*, and that further investigations are needed to characterize the effect of different physiologic conditions on resistance. Since DR0070 was resistant to desiccation, it is possible that genes involved in resistance to acute radiation are distinct from those involved in desiccation resistance. Mattimore and Battista (1996) have shown that some genes in *D. radiodurans* needed to survive irradiation are also necessary for desiccation resistance. However, a recent report by Battista and coworkers (2001) has shown the existence of genes in *D. radiodurans* that affect desiccation resistance but not radiation resistance, indicating that resistance to these conditions may involve different mechanisms as proposed here.

DR0105 has tentatively been given a functional assignment as a desiccation resistance protein of the Lea-76 family (Makarova K. S., *et al*., 2001). The Lea-76 family belongs to group 3 of the LEA proteins, which are well-characterized and widespread desiccation-induced proteins in plants. Besides plants, at least two proteins of this family are present in the nematode *Caenorhabditis elegans*. The Lea-76 motif is conserved in two hypothetical *D. radiodurans* desiccation proteins, DR0105 and DR1172. DR0105 was disrupted by tandem duplication (Fig. 2.1), shown to be homozygous, and was tested for resistance to γ radiation (Table 2.1), its ability to grow at 37°C (Fig. 2.4) and survive desiccation (Fig. 2.12). The DR0105 mutant was resistant to radiation, desiccation, and growth at 37°C. The ability of mutant DR0105 to resist desiccation does not support that this gene is essential for desiccation resistance in *D. radiodurans.* However, the presence of DR1172 in *D. radiodurans* with overlapping Lea-76 functions could be masking any effect of a disrupted DR0105. Therefore, constructing a double mutant in DR0105 and DR1172 would be a useful approach in further defining the possible role of Lea-76 genes in desiccation resistance in *D. radiodurans*.

DR2339 is a representative of the LigT protein family, which is found in several bacteria, archae, and eukaryotes and includes RNA ligases and predicted 2'-5'cyclic nucleotide phosphodiesterases. In addition to the LigT ortholog (DR2339), *D. radiodurans* encodes two predicted phosphdiesterases of this family (DR1000 and DR1814) that may also participate in RNA metabolism or signaling. DR2339 was selected for disruption based on its elevated expression (14-fold) following acute radiation (Table 2.1). The gene has been disrupted by tandem duplication and the mutant was shown to be homozygous (Gaidamakova E. K., *et al*., 2003). Mutant DR2339 is sensitive to growth under chronic radiation, growth at 37°C, and desiccation, but resistant to acute radiation (Table 2.4). This supports that radiation resistance mechanisms responsible for survival under chronic radiation are related to those involved with growth at 37°C and desiccation resistance, but not to those involved with recovery from acute irradiation. Like the relationships observed between these phenotypes in DR0070 and DR0105, the resistance phenotype of DR2339 is complex. It is notable that DR2339 appears to be sensitive to genotoxic conditions associated with growth conditions. As such, a disrupted DR2339 could act by rendering the cell sensitive to the effects of

superoxide free radicals, which are produced at high levels during metabolism, but not significantly during radiolysis during acute radiation.

2.5. Conclusions

1. Results support that radiation resistance mechanisms responsible for survival following exposure to acute radiation are distinct from those needed for survival under chronic radiation. For example, whereas mutant DR0070 is sensitive to acute radiation but not chronic radiation, mutant DR2339 is sensitive to chronic radiation but not acute radiation.

2. The ability of mutant DR0105, encoding a predicted desiccation resistance protein, to resist desiccation does not support that it is essential for desiccation resistance in *D. radiodurans.* However, other predicted desiccation resistance proteins in *D. radiodurans* may have overlapping functions that could mask the effect of a disrupted DR0105.

3. Results support that radiation resistance mechanisms responsible for survival under chronic radiation are related to those involved with growth at 37°C and desiccation resistance. For example, mutant DR2339 is sensitive to chronic radiation, growth at 37°C, and desiccation, but not acute radiation.

Chapter 3: Construction and Characterization of Double Mutants

3.1. Introduction

While the damaging effects of reactive oxygen radicals generated by ionizing radiation are well-documented (Gutteridge J. M. C., Halliwell B., 1999), relatively little is known about the complexity of genetic mechanisms in bacteria or eukaryotes involved in recovery from radiation. The previous chapter illustrates the difficulty in interpreting the phenotypes of mutants. For example, the finding that disruption of DR0105, encoding a predicted desiccation resistance protein, did not render the mutant sensitive to desiccation does not rule out the possibility that DR0105 is involved in desiccation resistance. Instead it is possible that other desiccation resistance genes may complement its disrupted function (*e.g.*, DR1172). As such, the construction of double mutants may be a useful approach in defining the role of genes where multiple genes with overlapping functions exist. The approach to constructing double mutants exploits *D. radiodurans'* natural transformation ability in combination with the existence of transforming constructs tagged with different antibiotic resistance markers. Typically a transforming construct encodes Km^R, but others encode Cm^R, which enables the construction of double mutants by transformation and double antibiotic selection. To investigate the possibility that two genes with overlapping function need to be knocked out to yield a sensitive phenotype, several distinct pairs of genes were selected for disruption in the same strain according to the scheme presented in Fig. 3.1. Transformants were analyzed for their ability to survive acute and chronic doses of ionizing radiation.

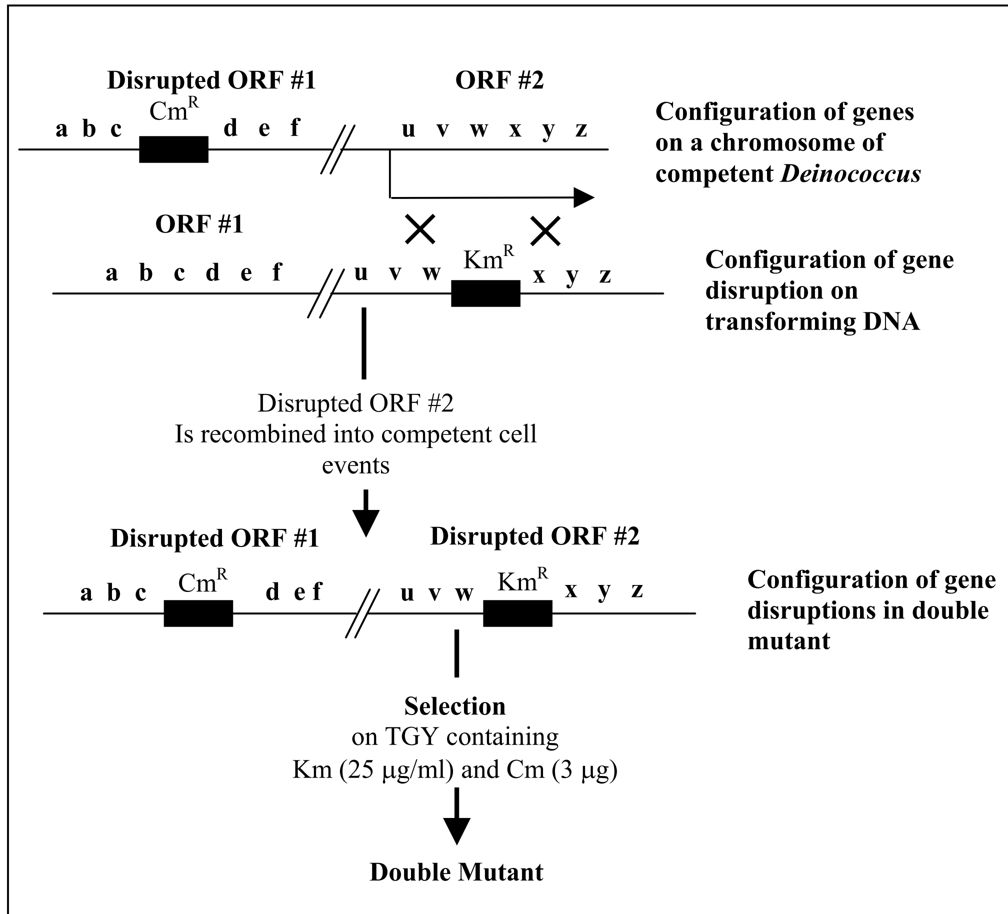
Disrupting two uncharacterized genes which lack predicted overlapping functions in the same host explores the possibility that distinct genes can share the same function. Because the premise of this approach is much like a 'fishing expedition', the likelihood of identifying functional equivalence in non-related genes is low. An alternative approach would be to disrupt all genes sharing a similar predicted function. In the case of predicted desiccation resistance genes (*e.g.*, DR0105), there are at least five in *D. radiodurans*, too many to disrupt at the same time given current genetic technology.

In *D. radiodurans*, there are only two genes predicted to function as DNA ligases, DR2069 and DRB0100. DNA ligases are enzymes required for repair, replication and recombination of DNA and are divided into two groups based on their requirement of specific cofactors. DR2069 is predicted to encode a NAD-dependent (bacterial type) DNA ligase, whereas DRB0100 encodes a predicted ATP-dependent (eukaryotic/archaeal-type) DNA ligase (Doherty A. J., Suh S. W., 2000). Analysis of radiation resistance for the two single mutants showed that the single heterozygous mutant DR2069 and the single mutant DRB0100 are both radiation resistant. A double mutant for DRB0100 and DR2069 was constructed and evaluated for radiation resistance. Specific aims of this chapter are as follows:

1. To construct *D. radiodurans* double mutants each containing two distinct disrupted novel genes implicated in recovery.

2. To determine the role of two different *D. radiodurans* DNA ligases in the extreme resistance phenotype.

Figure 3.1. Approach to constructing *D. radiodurans* double mutants



Legend Fig. 3.1. A Competent cell with a disruption in ORF #1 (**a b c-[~~ORF #1~~-Cm^R]-d e f**) and a wild-type genomic sequence #2 (**u v w-ORF #2-x y z**) is transformed with genomic DNA from a strain with a disrupted ORF #2 (**u v w -[~~ORF #2~~-Km^R]-x y z**). ORF disruptions (black boxes) are described in detail in Fig. 2.1. Two homologous recombination events (crossover) are indicated by dark crosses (**X** **X**). Since *D. radiodurans* contains 4-8 haploid copies of its genome, Cm^R/Km^R double transformants are subjected to extended selection with Cm and Km to force cells to homozygosity.

3.2. Materials and Methods

3.2.1. Competent *D. radiodurans* Cells and Transformation

Single colonies of *D. radiodurans* mutants pre-grown at 32˚C on TGY (containing appropriate antibiotics for selection), were transferred to 5 ml TGY containing either Km or Cm and grown at 32˚C with aeration to OD_{600} = 0.9. Cell density was determined by measuring OD_{600} as described in Chapter 2. Cells were harvested by centrifugation (5 min, 1,331 x *g*, 4˚C) and each pellet was resuspended in 7.7 ml filter-sterilized TGY/$CaCl_2$/glycerol (65% (v/v) TGY/ 25 mM $CaCl_2$ / 1% glycerol). Aliquots containing 250 µl of the suspension were frozen immediately in dry ice and stored at –70˚C.

Competent cells were thawed at 37˚C for 1 min and placed on ice. 100 µl of competent cells were added to i) 5 µl genomic *D. radiodurans* DNA, ii) 5 µl dH_2O (no-DNA control), and iii) 1 µl pMD68 (transforming plasmid control). Contents were mixed gently, placed on ice for 10 min, and then incubated for 30 min at 32˚C with aeration. One ml TGY containing antibiotic was subsequently added to each of the cell incubations. The cell mixtures were incubated for 16 h (32˚C) with aeration, before 100 µl of appropriately diluted samples were transferred to drug selective TGY agar plates and incubation at 32˚C. CFU counts were determined after three days. Five representative transformed colonies were further selected in media containing antibiotic for three days (32˚C). After extended selection, a stock culture of each clone was prepared as described in section 3.2.5. For clones under continuous laboratory culture, cells were subcultured weekly on fresh selective medium.

3.2.2. Isolation of Genomic DNA

Single colonies of *D. radiodurans* mutants pre-grown at 32˚C on selective TGY plates, were transferred to 5 ml selective TGY and grown at 32˚C with aeration to OD_{600} = 1.0. DNA was prepared by resuspending cells in 570 µl sterile TE buffer (10 mM Tris-HCl; 1 mM EDTA; pH 8) plus 15 µl of 20% SDS (Quality Biological Inc., MD) and 200 µg proteinase K ([20 mg/ml], Sigma, MO) followed by incubation at 37˚C for 1 h. 125 µl of 4 M NaCl and 80 µl CTAB/NaCl (10% (w/v) CTAB (Hexadecytrimethyl ammonium bromide); 4.1% (w/v) NaCl; in dH_2O) were then added and the mixture incubated at 65˚C

for 10 min to lyse the cells. Genomic DNA was purified by adding 800 μl chloroform:isoamylalcohol (24:1) mix, followed by centrifugation at 9,400 x *g* for 10 min at room temperature to separate the aqueous and organic phases. The upper aqueous phase, containing the genomic DNA, was re-extracted with an equal volume phenol:chloroform:isoamylalcohol (25:24:1) mix, followed by vortexing and centrifugation at 9,400 x *g* for 10 min. The aqueous phase was transferred to a 1.5 ml Eppendorf tube and isopropanol was added to 60% (v/v). After mixing, the DNA was allowed to precipitate. DNA was isolated by centrifugation and then air-dried before dissolving it in 100 μl of TE buffer (Quality Biological Inc., MD). Genomic DNA was stored at -20˚C. To obtain RNA-free genomic DNA, 50 μl of each genomic DNA sample was treated with 1 μl DNase-free RNase (500 ng/μl, Roche, CH) according to the manufacture's instructions, followed by phenol/chloroform extraction and precipitation with ethanol. RNA-free genomic *D. radiodurans* DNA was stored at -20˚C.

3.2.3. Quantitative Analysis of DNA by Spectrofluorometry

To calculate the concentration of DNA samples, 5 μl of purified DNA were transferred to 995 μl dH_2O containing 1 ml 2 x SYBR Gold nucleic acid gel stain (Molecular Probes Inc., OR). The extinction of DNA solution was measured at λ = 494 nm in a calibrated SLM Aminco SPF – 500 CTM spectrofluorometer (SLM Instruments Inc., NY).

3.2.4. Qualitative Analysis of DNA by Gel Electrophoresis

Two μl of RNA-free DNA were diluted in 8μl TE buffer and 2 μl gel loading buffer [0.25% (w/v) bromophenol blue (Sigma, MO), 0.25% (w/v) xylene cyanol FF, 15% (w/v) Ficoll (Type 400; Amersham, NJ) in dH_2O]. The mixture was loaded on a 0.66% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide (Sigma, MO), with TAE Tris acetate; Quality Biological Inc., MD) as the running buffer. Molecular weight markers were included. Condition for electrophoresis: 96 V for 2.5 h at room temperature. The gel was analyzed on an Eagle Eye II illuminator using Eagle Eye software (Stratagene, CA).

3.2.5. Storage of Transformants

Single colonies of the transformed clones were inoculated to10 ml double Cm + Km TGY. Inoculates were incubated with aeration at 32˚C to OD_{600} = 1. Cells were harvested by centrifugation. After washing in TGY, cells were re-suspended in sterile 50% glycerol and stored at -80˚C.

3.2.6. Irradiation Survival Measurements

Single colonies of wild-type *D. radiodurans* (R1), single mutants, and double mutants (Table 3.1) were transferred to liquid TGY containing the indicated antibiotics (Table 3.1) and grown to OD_{600} ~0.9. For irradiation, 60 µl of each culture were transferred to a 0.65 ml Eppendorf tube. An unirradiated control culture was maintained on ice for the duration of the irradiation. For high-level acute radiation, cells were maintained on ice at 8 kGy/h. 20 µl of each culture were removed and placed on ice after 3, 5, and 9 kGy. About 5 µl of irradiated and non-irradiated cell samples were transferred to TGY plates containing Km (25 µg/ml) or Km (25 µg/ml) plus Cm (3 µg/ml) using sterile technique. Regions of plates inoculated with cells were examined visually after 3 days for evidence of growth. Ability of mutants to grow under chronic radiation is as described in Chapter 2.

Table 3.1. Strains

***D. radiodurans* strain**	**Antibiotic concentration**
R1 (wild-type)	none
MD68 Km^R (plasmid control)	25 μg/ml Km
[a]**MD424 Km^R-Cm^R (chromosome control)**	25 μg/ml Km 3 μg/ml Cm
$DR0070^-$ Cm^R	3 μg/ml Cm
$DR2482^-$ Cm^R	3 μg/ml Cm
$DRB0100^-$ Cm^R	3 μg/ml Cm
$DR0070^-$ Km^R	25 μg/ml Km
$DR0105^-$ Km^R	25 μg/ml Km
$DR2069^-$ Km^R	25 μg/ml Km
$DR2339^-$ Km^R	25 μg/ml Km
[b]**DM ($DR0070^-Cm^R$ + $DR0105^-Km^R$)**	25 μg/ml Km 3 μg/ml Cm
[b]**DM ($DR0070^-Cm^R$ + $DR2069^-Km^R$)**	25 μg/ml Km 3 μg/ml Cm
[b]**DM ($DR2482^-Cm^R$ + $DR0070^-Km^R$)**	25 μg/ml Km 3 μg/ml Cm
[b]**DM ($DR2482^-Cm^R$ + $DR0105^-Km^R$)**	25 μg/ml Km 3 μg/ml Cm
[b]**DM ($DRB0100^-Cm^R$ + $DR0070^-Km^R$)**	25 μg/ml Km 3 μg/ml Cm
[b]**DM ($DRB0100^-Cm^R$ + $DR2069^-Km^R$)**	25 μg/ml Km 3 μg/ml Cm
[b]**DM ($DRB0100^-Cm^R$ + $DR2339^-Km^R$)**	25 μg/ml Km 3 μg/ml Cm

Footnotes Table 3.1

[a]MD424 is a radiation resistant strain encoding Km and Cm on Chromosome I.

[b]DM, double mutant construction.

3.3. Results

3.3.1. Transformation

For all DNA transformations into *Deinococcus*, DNA was purified by phenol/chloroform extraction and examined by agarose gel electrophoresis (AGE). AGE showed that all transforming DNA preparations were non-degradated and had high molecular weights (Fig. 3.2). Concentrations of purified DNA are listed in Table 3.2. Transformation of *D. radiodurans* Cm^R-single mutants (Table 2.1) with DNA from Km^R-single mutants (Table 2.1) generated 55 clones representing 21 distinct double mutant combinations/constructions (Table 3.3-3.5) capable of growth on double Km + Cm selective agar. Double mutant candidates were archived in the USUHS *Deinococcus* collection under the names listed in Table 3.3-3.5. Seven double mutant combinations represented as 19 clones were further investigated (Table 3.6)

Table 3.2. Measurement of DNA concentration

***D. radiodurans* Mutant**	**DNA Concentration [µg/ml]**
DR0070	36
DR0105	1,237
DR0140	37
DR1916	49
DR2069	1,010
DR2339	1,200
DR2482	53
DRA0145	47
DRB0100	1,886
DRC0012	102

Footnote Table 3.2. DNA concentration was determined by transferring extinction of calibration dilutions (λ DNA) and of the deinococcal DNA samples to one graph.

Figure 3.2. Analysis of genomic DNA by gel electrophoresis

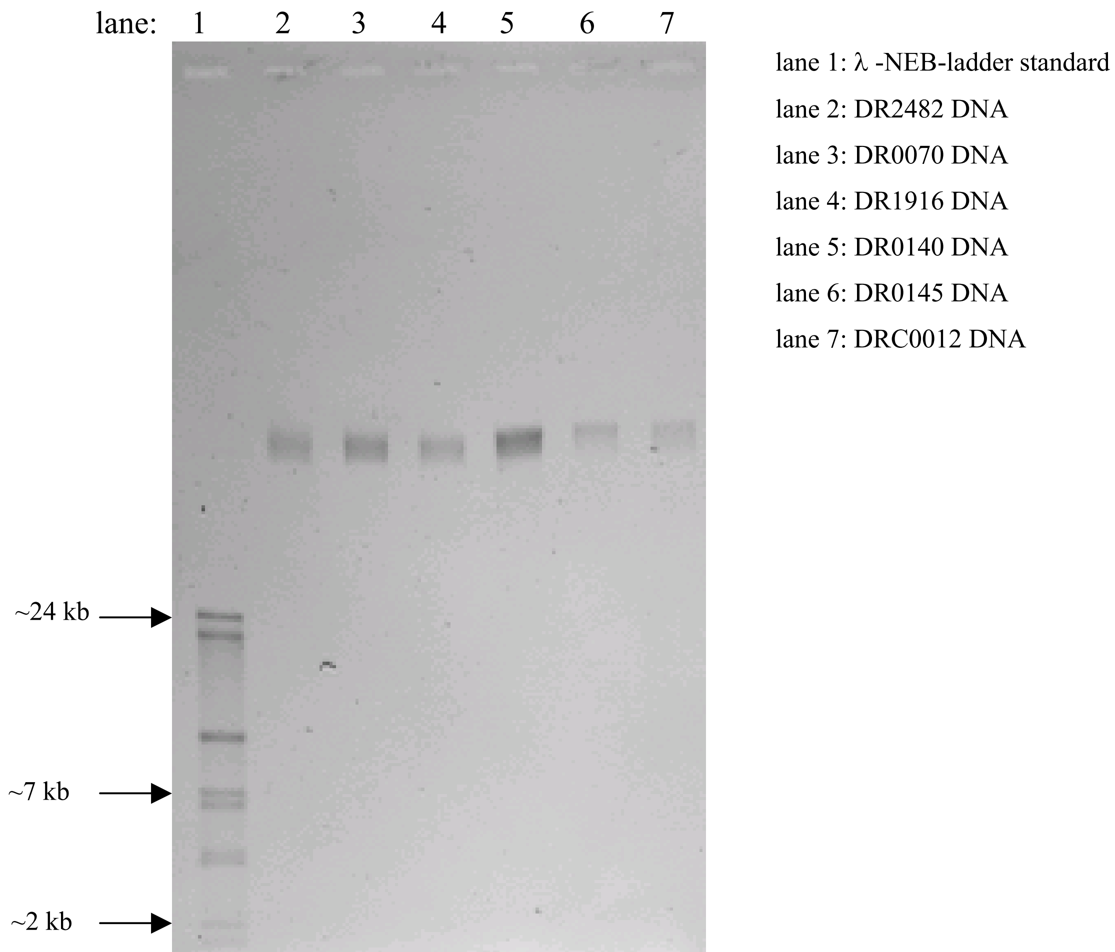


Table 3.3. Double mutant constructions derived from mutant DR0070

Name of strains/construct	Double mutant construction	Gene ID/predicted function of gene[b]
MH1	[a]DM (DR0070 + DR2482)	DR2482/ Sigma factor
MH2	[a]DM (DR0070 + DR2482)	
MH3	[a]DM (DR0070 + DR0105)	DR0105/ Desiccation resistance protein
MH4	[a]DM (DR0070 + DR0105)	
MH5	[a]DM (DR0070 + DR1916)	DR1916/ RecG helicase
MH6	[a]DM (DR0070 + DR1916)	
MH7	[a]DM (DR0070 + DR0140)	DR0140/ Uncharacterized protein
MH8	[a]DM (DR0070 + DR0140)	
MH9	[a]DM (DR0070 + DRA0145)	DRA0145/ Peroxidase
MH10	[a]DM (DR0070 + DRA0145)	
MH11	[a]DM (DR0070 + DRB0100)	DRB0100/ Homolog to eukaryotic DNA ligase III
MH12	[a]DM (DR0070 + DRB0100)	
MH13	[a]DM (DR0070 + DRC0012)	DRC0012/ Transcriptional regulator
MH14	[a]DM (DR0070 + DRC0012)	
MH15	[a]DM (DR0070 + DR2069)	DR2069/ NAD^+ dependant DNA ligase
MH16	[a]DM (DR0070 + DR2069)	
MH17	[a]DM (DR0070 + DR2069)	

Footnotes Table 3.3

[a]DM, double mutant construction.

[b]Gene ID and of predicted function of targeted gene (Makarova K. S., *et al*., 2001).

Table 3.4. Double mutant constructions derived from mutant DR2482

Name of strain/construct	Double mutant construction	Gene ID/ predicted function of gene[b]
MH18	[a]DM (DR2482 + DR0070)	DR0070/ Alkaline protease
MH19	[a]DM (DR2482 + DR0070)	
MH20	[a]DM (DR2482 + DR0105)	DR0105/ Desiccation resistance protein
MH21	[a]DM (DR2482 + DR0105)	
MH22	[a]DM (DR2482 + DR1916)	DR1916/ RecG helicase
MH23	[a]DM (DR2482 + DR1916)	
MH24	[a]DM (DR2482 + DR0140)	DR0140/ Uncharacterized protein
MH25	[a]DM (DR2482 + DR0140)	
MH26	[a]DM (DR2482 + DRA0145)	DRA0145/ Peroxidase
MH27	[a]DM (DR2482 + DRA0145)	
MH28	[a]DM (DR2482 + DRB0100)	DRB0100/ Homolog to eukaryotic DNA ligase III
MH29	[a]DM (DR2482 + DRB0100)	
MH30	[a]DM (DR2482 + DRC0012)	DRC0012/ Transcriptional regulator
MH31	[a]DM (DR2482 + DRC0012)	

Footnotes Table 3.4 are as in Table 3.3.

Table 3.5. Double mutant constructions derived from mutant DRB0100

Name of strain/construct	Double mutant construction	Gene ID/ predicted function of gene[b]
MH32	[a]DM (DRB0100 + DR2482)	DR2482/ Sigma factor
MH33	[a]DM (DRB0100 + DR2482)	
MH34	[a]DM (DRB0100 + DR0070)	DR0070/ Alkaline protease
MH35	[a]DM (DRB0100 + DR0070)	
MH36	[a]DM (DRB0100 + DR0105)	DR0105/ Desiccation resistance protein
MH37	[a]DM (DRB0100 + DR0105)	
MH38	[a]DM (DRB0100 + DR1916)	DR1916/ RecG Helicase
MH39	[a]DM (DRB0100 + DR1916)	
MH40	[a]DM (DRB0100 + DR0140)	DR0140/ Uncharacterized protein
MH41	[a]DM (DRB0100 + DR0140)	
MH42	[a]DM (DRB0100 + DRA0145)	DRA0145/ Peroxidase
MH43	[a]DM (DRB0100 + DRA0145)	
MH44	[a]DM (DRB0100 + DRC0012)	DRC0012/ Transcriptional regulator
MH45	[a]DM (DRB0100 + DRC0012)	
MH46	[a]DM (DRB0100 + DR2069)	DR2069/ NAD^+ dependant DNA ligase
MH47	[a]DM (DRB0100 + DR2069)	
MH48	[a]DM (DRB0100 + DR2069)	
MH49	[a]DM (DRB0100 + DR2069)	
MH50	[a]DM (DRB0100 + DR2069)	
MH51	[a]DM (DRB0100 + DR2339)	DR2339/ 2'-5' RNA ligase
MH52	[a]DM (DRB0100 + DR2339)	
MH53	[a]DM (DRB0100 + DR2339)	
MH54	[a]DM (DRB0100 + DR2339)	
MH55	[a]DM (DRB0100 + DR2339)	

Footnotes Table 3.5 are as in Table 3.3.

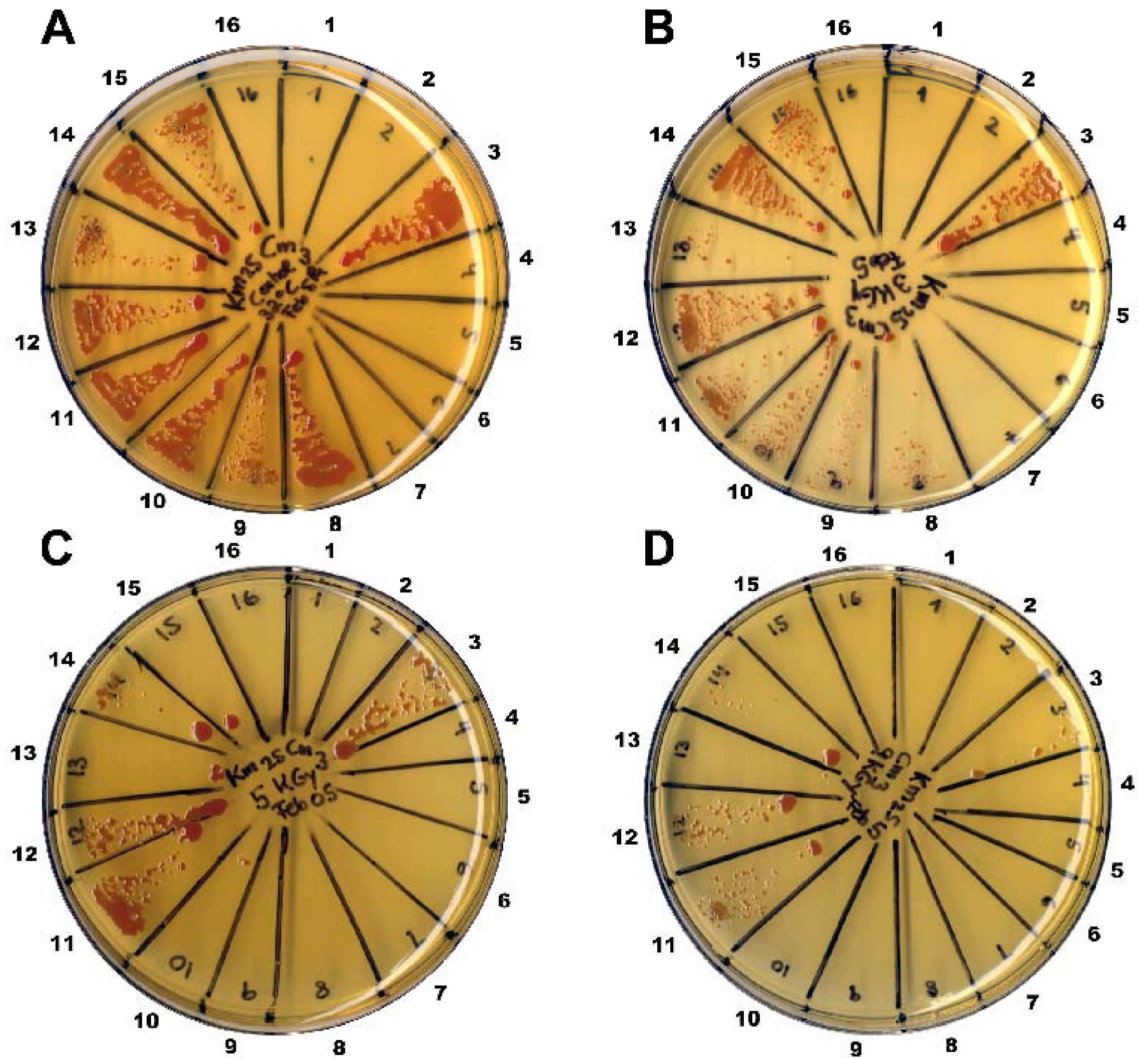
3.3.2. Growth under Chronic Radiation

None of the 55 transformants capable of growth on Cm + Km agar plates were able to grow under chronic radiation, including the wild-type control MD424 (Km^R-Cm^R) (data not shown). Therefore, resistance of the double mutants to chronic radiation could not be determined.

3.3.3 Recovery from Acute Radiation

All non irradiated double mutants grew in double selective (Km Cm) TGY, and a decrease of growth was observed for all strains following high doses of acute radiation (Figure 3.3). As expected, the control strains lacking resistance to both Km and Cm did not grow on double antibiotic plates irrespective of radiation. The greatest reduction in growth at increased radiation levels was for clones of group [DR0070 + DR2069] (Figure 3.3). Of the seven double mutant combinations investigated (Table 3.6), only clones of group [DRB0100$^-$$Cm^R$ + DR2069$^-$$Km^R$] showed a relative increase in sensitivity to acute radiation compared to the individual mutant parental strains (Table 2.1). An example showing the results of the acute radiation resistance assay for clones representing the DRB0100$^-$$Cm^R$ + DR2069$^-$$Km^R$ construction is shown in Fig. 3.3. Since the parental strains of MH48, MH49, and MH50 (DRB0100$^-$$Cm^R$ + DR2069$^-$$Km^R$) (Table 3.6) were resistant to high doses of acute radiation, but the double mutant derivatives were not, this group of predicted DNA ligase mutants was further investigated in Chapter 4.

Figure 3.3. Recovery from acute radiation



Legend Fig. 3.3

Plate A, TGY agar, no irradiation (control).

Plate B, inoculated as in plate A with cells exposed to 3 kGy.

Plate C, inoculated as in plate A with cells exposed to 5 kGy.

Plate D, inoculated as in plate A with cells exposed to 9 kGy.

Plate sections 1-16 contained the indicated strains: 1) R1, 2) MD68, 3) MD424, 4) DR0070, 5) DR0070^{-}Cm^{R}, 6) DRB0100^{-}Cm^{R}, 7) DR2069, 8) MH15, 9) MH16, 10) MH17, 11) MH46, 12) MH47, 13) MH48, 14) MH49, 15) MH50. 16) No sample. See Table 3.1, 3.3 and 3.5 for strain descriptions.

Table 3.6. Acute radiation resistance phenotype of double mutant constructions

Name of construct	Double mutant construction	Acute radiation resistance phenotype compared to parental strains
MH3	[a]DM ($DR0070^-Cm^R$ + $DR0105^-Km^R$)	same
MH4	[a]DM ($DR0070^-Cm^R$ + $DR0105^-Km^R$)	same
MH15	[a]DM ($DR0070^-Cm^R$ + $DR2069^-Km^R$)	same
MH16	[a]DM ($DR0070^-Cm^R$ + $DR2069^-Km^R$)	same
MH17	[a]DM ($DR0070^-Cm^R$ + $DR2069^-Km^R$)	same
MH18	[a]DM ($DR2482^-Cm^R$ + $DR0070^-Km^R$)	same
MH19	[a]DM ($DR2482^-Cm^R$ + $DR0070^-Km^R$)	same
MH20	[a]DM ($DR2482^-Cm^R$ + $DR0105^-Km^R$)	same
MH21	[a]DM ($DR2482^-Cm^R$ + $DR0105^-Km^R$)	same
MH34	[a]DM ($DRB0100^-Cm^R$ + $DR0070^-Km^R$)	same
MH35	[a]DM ($DRB0100^-Cm^R$ + $DR0070^-Km^R$)	same
MH46	[a]DM ($DRB0100^-Cm^R$ + $DR2069^-Km^R$)	same
MH47	[a]DM ($DRB0100^-Cm^R$ + $DR2069^-Km^R$)	same
MH48	[a]DM ($DRB0100^-Cm^R$ + $DR2069^-Km^R$)	more sensitive
MH49	[a]DM ($DRB0100^-Cm^R$ + $DR2069^-Km^R$)	more sensitive
MH50	[a]DM ($DRB0100^-Cm^R$ + $DR2069^-Km^R$)	more sensitive
MH51	[a]DM ($DRB0100^-Cm^R$ + $DR2339^-Km^R$)	same
MH52	[a]DM ($DRB0100^-Cm^R$ + $DR2339^-Km^R$)	same
MH53	[a]DM ($DRB0100^-Cm^R$ + $DR2339^-Km^R$)	same

Footnote Table 3.6
[a]DM, double mutant construction.

3.4. Discussion

The lack of a clearly identifiable unique DNA repair system in *D. radiodurans* has given rise to several competing views of the mechanisms responsible for its extraordinary survival (Chapter 1). This chapter builds on recent results from this laboratory showing that few of the identified novel genes of *D. radiodurans* appear to contribute to radiation resistance (Liu Y., *et al*., 2003; Gaidamakova, E. K., *et al*., 2003). To date, only two of about 40 distinct single mutants examined show sensitivity to radiation (DR0070 & DR2339, Chapter 2). This finding suggests that novel repair functions encoded among hypothetical genes predicted by genomic annotation may have overlapping functions. If this were the case, two or more genes with related function would need to be disrupted to obtain a radiation sensitive phenotype. The goal of this chapter was to examine the radiation resistance characteristics of i) *D. radiodurans* double mutants each containing two distinct disrupted novel genes, and ii) a *D. radiodurans* strain disrupted in two different, but related DNA ligases.

Transformation of the Cm-resistant knockout mutants DRB0100, DR2482, and DR0070 with genomic DNA from other Km-resistant single mutant strains (Table 3.3-3.5) generated 55 clones capable of growth in the presence of Km and Cm double selection (Table 3.3-3.5). A preliminary characterization of the radiation resistance profiles of these strains is presented (Fig. 3.3, Table 3.6). Since this thesis work represents only the foundation for future investigations, all strains reported here have been archived in the USUHS *Deinococcus* frozen collection. Nineteen representative strains (Table 3.6) were exposed to acute and chronic ionizing radiation to determine if their resistance phenotype was different from either of the parental strains (Table 2.1).

The first general observation was that while the control strain (MD424), with chromosomally encoded Km and Cm resistance, could grow at 22°C in the presence of double antibiotic selection, none of the double mutant constructs could grow at 22°C (data not shown). This suggests that the single and double mutant combinations were significantly less robust than wild-type in resisting the stress of antibiotic selection. However, only three of the 55 Cm/Km-resistant isolates showed decreased resistance to acute radiation compared to their respective parental strains (Table 3.6), and none of the prospective double mutants could grow under chronic radiation in the presence of both

Cm and Km. However, the wild-type control strains expressing Cm^R and Km^R could also not grow under chronic radiation.

The three sensitive clones sensitive to acute radiation were all derived from the DRB0100 (eukaryotic-type DNA ligase) x DR2069 (bacterial-type DNA ligase) double mutant construction. These three strains (MH48-50, Table 3.6) showed increased sensitivity to acute 9 kGy (Fig. 3.3) when compared to the parental strains DRB0100 and DR2069. The increased sensitivity of strains MH48-50 [$DRB0100^-Cm^R$ + $DR2069^-Km^R$] (Table 3.6) justified further genetic characterization of the DNA ligase disruptions and their possible role in the resistance phenotype (Chapter 4).

3.5. Conclusion

Fifty-five *D. radiodurans* clones capable of growth on Cm + Km double selection were isolated, and represent 19 double mutant combinations (Table 3.3-3.5). Only one double mutant group DM ($DRB0100^-Cm^R$ + $DR2069^-Km^R$) was relatively sensitive to acute doses of irradiation compared to the parental strains, which are disrupted in the predicted eukaryotic and bacterial DNA ligases of *D. radiodurans* (Table 2.1 & 3.6).

Chapter 4: Genetic Characterization of DNA Ligase Double Mutants

4.1. Introduction

Annotation for the whole genome sequence of *D. radiodurans* identified the presence of two distinct DNA ligases (Makarova K. S., *et al*., 2001) encoded by DR2069 (bacterial) and DRB0100 (eukaryotic) (Chapter 3). DRB0100 and DR2069 were both previously subjected to insertional inactivation (Gaidamakova E. K., *et al*., 2003). Whereas mutant DRB0100 was shown to be homozygous, DR2069 was persistently heterozygous irrespective of antibiotic selection. Therefore, DRB0100 is considered to be a non-essential gene, but DR2069 likely is essential. Phenotypic analysis of the single mutants DRB0100 and DR2069 showed that both have wild-type radiation resistance characteristics. However, since there is at least one wild-type copy of DR2069 in the DR2069 mutant, it is not possible to conclude that the gene does not have a role in the resistance phenotype. In *D. radiodurans*, expression levels for genes are positively correlated with their copy number (Brim H., *et al*., 2000); *i.e*., the higher the copy number, the higher the expression level. If this is the case for the heterozygous mutant DR2069 (assuming three mutant copies and one wild-type copy, based on autoradiography (Gaidamakova E. K., *et al*., 2003), it may have reduced levels of the bacterial-type DNA ligase. Yet, the resistance phenotype of the single mutant was the same as wild-type. However, transformation of DRB0100$^-$Cm^R cells with genomic DNA from DR2069$^-$Km^R yielded clones that were significantly more sensitive to acute radiation than either of the parental strains. This suggests that possible overlapping functions of the DNA ligases may be important to the resistance phenotype of *D. radiodurans* (Chapter 3).

To confirm that the radiation sensitive phenotype of the [DRB0100$^-$Cm^R + DR2069$^-$Km^R] constructs were the result of disruptions in both DNA ligases, clones were subjected to a detailed genetic mapping. Prospective double mutants were subjected to Southern analysis using diagnostic radio-labeled probes, that were used to generate a detailed map of the integration sites of DRB0100 and DR2069. The specific aim of this

chapter was to determine if ORFs DR2069 and DRB0100 are disrupted in the radiation sensitive DNA ligase constructs.

4.2. Material and Methods

4.2.1. Extraction of Genomic DNA

DNA was isolated from wild-type strain R1 and five clones of the double mutant group ($DRB0100^{-}Cm^{R}$ + $DR2069^{-}Km^{R}$) (MH46-50) (Table 3.6) as described earlier.

4.2.2. Qualitative Analysis of DNA by Gel Electrophoresis

Isolated DNA was diluted as described in Chapter 3. One μl of 100 ng/ml, 50 ng/ml, and 20 ng/ml λ-DNA sample were diluted in 8 μl TE (pH 8) and 2 μl gel loading buffer. The mixture was loaded on a 0.66% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide, with Tris-borate EDTA (TBE; Quality Biological Inc., MD) as the running buffer. Conditions for electrophoresis were: 23 V, 14 h running at room temperature.

4.2.3. Digestion of Genomic *D. radiodurans* DNA

Isolated DNA was digested by either *Alw*I, *Ava*I, *Bsa*I, or *Hinc*II. All restriction enzymes and digestion buffers (Table 4.1) were purchased from NEB (New England Biolabs), MA. For each enzyme a master mix was prepared by mixing following: 21 μl dH_2O, 7 μl of 10 x restriction enzyme buffer, 1μl RNase, and 7 μl restriction enzyme. Five μl of the appropriate restriction master mix were added to 5 μl of isolated DNA. Mixtures were incubated for 1 h at the indicated temperature (Table 4.1). Digested DNA samples (10 μl/lane) and a DNA size standard (0.5 μl of 2-log DNA ladder (NEB, MA) [1,000 μg DNA/ml] mixed with 9.5 μl dH_2O and 2 μl gel loading buffer) was loaded on a 1% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide, with TBE as the running buffer. Gel electrophoresis was performed at 23 V for 14 h.

Table 4.1. DNA-digestion

Endonuclease	Recognition sequence (5' to 3' end)[a]	Buffer	Incubation temperature
*Alw*I	GGATC [b]	10 x NEB buffer 4	37˚C
*Ava*I	CYCGRG [b]	10 x NEB buffer 4	37˚C
*Bsa*I	GGTCTC [b]	10 x NEB buffer 3	50˚C
*Hinc*II	GTYRAC [b]	10 x NEB buffer 3	37˚C

Footnotes Table 4.1

[a]Sequence which is recognized and cut by the indicated enzyme (New England Biolabs, MA).

[b]A, C, G, and T indicate adenine, cytosine, guanine, and thymine, respectively. R indicates a purine and Y a pyrimidine.

4.2.4. Polymerase Chain Reaction

Primer "49 forward" and "49 reverse" (Table 4.2) was used to synthesize probe for gene DR2069 (Y. Liu at Oak Ridge National Laboratory, TN) and primer eg-00093 and eg-00094 were used to synthesize the diagnostic probe for DRB0100 (E. Gaidamakova, USUHS, MD).

Table 4.2. Primers for PCR probe-synthesis for DR2069 and DRB0100

Primer Name	Primer Sequence[a]	T_m[b]	GC Content[c]	Product length[c]
49 forward	5'-GTC AAA TAC CCT TCC ATC CCC C-3'	77.3˚C	54.4%	582 bp
49 reverse	5'-GAC TCA GCC AGT GCT GAT CGG T-3'	79.2˚C	59.1%	
eg-00093	5'-GAA GTG CGC GGC GAG GTC TAC-3'	63.5˚C	66.7%	907 bp
eg-00094	5'-GGC GCT GAG GGT ATA GAG GC -3'	60.3˚C	65%	

Footnotes Table 4.2

[a]A= adenine, C= cytosine, G= guanine, T= thymine

[b]T_m= Melting temperature (Integrated DNA Technologies, IA).

[c]Values were determined using Oligo Primer Analysis Software.

Primers for amplification of the kanamycin resistance gene (*aphA*) located on pCR2.1 (Table 4.3), were chosen by using Oligo Primer Analysis Software (Molecular Biology Insights, Inc., CO) and ordered from Integrated DNA Technologies, Inc., IA. Two µl of each primer [10 pmol/µl] were mixed, while kept on ice, with 25 µl 2 x PCR Master Mix (MBI Fermentas, MD), 5 µl pCR2.1 DNA (0.025 µg/µl) and 16 µl dH_2O. The PCR was performed in a GeneAmp PCR system 2700 (Applied Biosystems, CA) and conditions used were as follows:

Step 1 (denaturation)	: 94˚C	5 min
Step 2 (denaturation)	: 94˚C	30 sec
Step 3 (annealing)	: 59˚C	30 sec
Step 4 (synthesis)	: 72˚C	30 sec
Step 5	: repeat step 2 to step 4 for 30 times	
Step 6 (synthesis)	: 72˚C	7 min
Step 7	: keep product on 4˚C until stored at –20˚C	

Table 4.3. Primers for PCR synthesis of Km-probe

Primer Name	Primer Sequence[a]	T_m[b]	GC Content[c]	Product length[c]
eg-00149	5'-TGG ATT GCA CGC AGG TTC TCC -3'	61.3˚C	57.1%	750 bp
eg-00150	5'-GCG ATG CGC TGC GAA T -3'	57.1˚C	62.5%	
eg-00149	5'-TGG ATT GCA CGC AGG TTC TCC -3'	61.3˚C	57.1%	750 bp
eg-00150	5'-GCG ATG CGC TGC GAA T -3'	57.1˚C	62.5%	

Footnotes Table 4.3 are as in Table 4.2.

To confirm the correct size of the PCR product (750 bp), 8 µl of the product were mixed with 2 µl gel loading buffer and electrophoreses in a 1.5% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide. 6 µl "ready to use" Gene Ruler 100 bp (MBI Fermentas, MD) [0.1 mg DNA/ml], and 5 µl of 2-log ladder [50 µg DNA/ml] diluted in TE and 1 µl

gel loading buffer were loaded in separate lanes on the gel. Gel electrophoresis was performed at 23 V for 14 h in 1 x TBE with 0.5 μg/ml ethidium bromide as the running buffer. The gel was analyzed as described earlier. DNA was isolated and purified from the agarose gel using a GenEluteTM Minus EtBr Spin Column (Sigma, MO) according to the manufacturer's protocol. To estimate the DNA concentration, 1 μl of the purified PCR-product was diluted in 4 μl TE (pH 8) and 1 μl gel loading buffer. The mixture was loaded on to a 0.66% (w/v) agarose gel containing 0.5 μg/ml ethidium bromide with TBE as the running buffer. For comparison DNA size markers were included in the gel and used to estimate the concentration of DNA by comparing the fluorescence. The purified PCR-product was stored at –20˚C.

4.2.5. Southern Hybridization

The agarose gels were incubated for 30 min in denaturation buffer (3 M NaCl, 0.4 M NaOH, in dH_2O), rinsed in dH_2O and incubated in 1 x neutralization buffer (pH 6.8; 0.1 M Na_2HPO_4, 90 mM $NaH_2PO_4 \cdot H_2O$, in dH_2O) for 30 min. Before membranes were placed on the transfer platform, the membranes were equilibrated for 30 min in transfer buffer (3 M NaCl, 8 mM NaOH). For downward transfer of DNA from the gel to a transfer-membrane, a TURBOBLOTTER™ Rapid Downward Transfer Systems kit (Schleicher & Schuell Inc., NH) was used. The transfer system was assembled as described for alkaline and neutral transfer in the manual. Transfer was complete in 3 h. Complete transfer of DNA from the gel was confirmed on an Eagle Eye II illuminator. The transfer membranes were washed in 1 x neutralizing buffer for 5 min at room temperature prior to fixation of the DNA to the membrane by exposure to 254 nm light (total exposure dose: 120 mJ/cm^2) in an UV crosslinker (HL-2000 HybriLinker, UVP Laboratory Products, CA). After polypropylene meshes were applied to support the membranes they were transferred to hybridization roller tubes (Thomas Scientific, NJ) and stored at –20˚C.

300 μl aliquots of sheared calf thymus DNA [10 mg/ml], 10 ml aliquots pre-hybridization buffer (4 ml 20 x SSPE, 2 ml 20% (v/v) SDS, 2 ml BLOTTO [10% (w/v) dried skim milk], 31 ml dH_2O) and 10 ml aliquots hybridization buffer (3 ml 20 x SSPE, 1.5 ml 20% (v/v) SDS, 1.5 ml BLOTTO [10% (w/v) dried skim milk], 4 ml 20% (w/v)

dextran sulfate, 20 ml dH_2O) were thawed in a 65˚C water bath. Calf thymus DNA was boiled for 5 min and put on ice for 2 min before it was added to both pre-hybridization and hybridization buffer. The mixture of calf thymus DNA and pre-hybridization buffer was transferred to the hybridization roller tube containing the *D. radiodurans* DNA fixed to a transfer membrane. The tube was placed in a HL-2000 HybriLinker and incubated at 65˚C for 3 h under constant rotation. $\alpha[^{32}P]$-dCTP (ICN, Biomedicals Inc, IL) was thawed at room temperature. Five µl of PCR generated probe was diluted in 1 µl 2-log DNA ladder [1 µg DNA/ml] and 39 µl dH_2O. Mixtures were boiled for 2 min and placed immediately on ice for 2 min. Five µl of $\alpha[^{32}P]$-dCTP [250 µCi/mmol] were added and the mixture was transferred to a tube containing a DNA labeling bead (Ready-To-Go™ DNA Labelling Beads (-dCTP), Amersham Pharmacia Biotech, NJ), which contained DNA polymerase for DNA synthesis. While the sample was incubated for 30 min at 37˚C the pre-hybridization buffer in the roller tube was discarded and the hybridization buffer-calf thymus DNA mixture was transferred to the roller tube. A ProbeQuant™ G-50 Micro Column (Amersham Pharmacia Biotech, NJ) was used to purify labeled probe from unincorporated labeled nucleotides according to the manufacturer's protocol. Denatured radiolabeled probe was transferred to the roller tube which contained hybridization buffer and the membrane. The tube was placed in a HL-2000 HybriLinker and incubated for 16 h at 65˚C. Hybridization buffer was removed and the membrane was washed twice for 30 min with wash-buffer #1 (0.5% (v/v) SDS, 2 x SSPE) followed by two washing steps in wash-buffer #2 (0.1% (v/v) SDS, 0.1 x SSPE). Both washing steps were performed at 60˚C. The membrane was removed from the tube, sealed in a plastic bag, and subjected to autoradiography using X-ray film (Kodak, NY). After exposure, the film was developed in a M35A X-OMAT Processor (Kodak, NY).

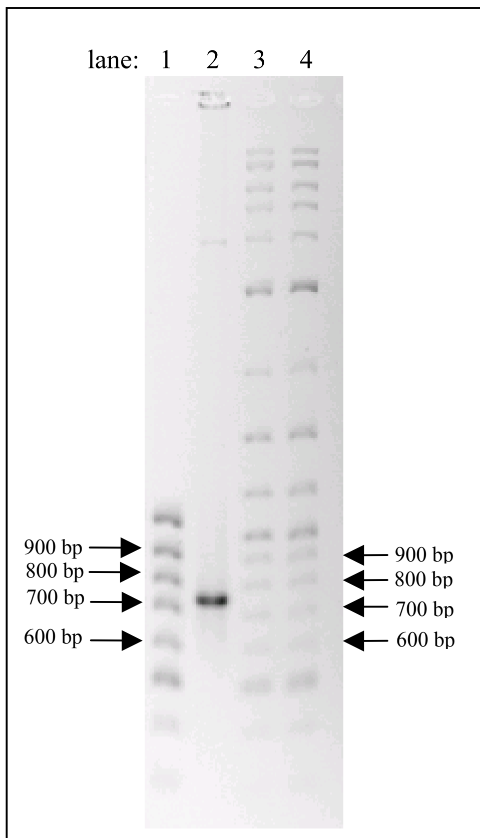
For re-hybridization with a different probe, the membrane was stripped in wash buffer #3 (0.2 M NaOH, 0.1% SDS) for 30 min at 37˚C.

4.3. Results

4.3.1. Polymerase Chain Reaction

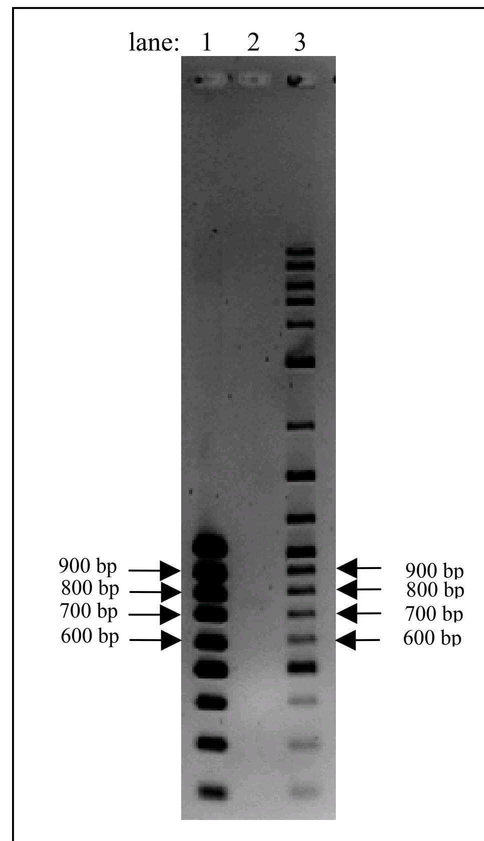
Amplification of part of the gene encoding kanamycin resistance (*aphA*) on pCR2.1 was used to generate the probe for mapping the DR2069 disruption. The amplification generated a ~750 bp PCR product (Figs. 4.1 & 4.2).

Figure 4.1. PCR-product sizing



Legend Fig. 4.1
lane 1: "ready to use" Gene Ruler 100 bp
lane 2: PCR-product
lane 3: 2-log ladder
lane 4: 2-log ladder

Figure 4.2. Gel-purified PCR-product



Legend Fig. 4.2
lane 1: "ready to use" Gene Ruler 100 bp
lane 2: Purified PCR-product
lane 3: 2-log ladder

4.3.2. DNA Isolation and Digestion

Gel electrophoresis showed that genomic (template) DNA was not degraded (Fig. 4.3). The concentration of genomic DNA was estimated to be about 10 ng/µl. Restriction endonuclease digested DNA was separated by agarose gel electrophoresis. (Figs. 4.4 & 4.5). As numerous digestion sites specific for each of the selected enzymes are present in the deinococcal genome numerous bands were observed after the agarose gel was analyzed (Fig. 4.4).

Figure 4.3. Qualitative analysis of DNA by gel electrophoresis

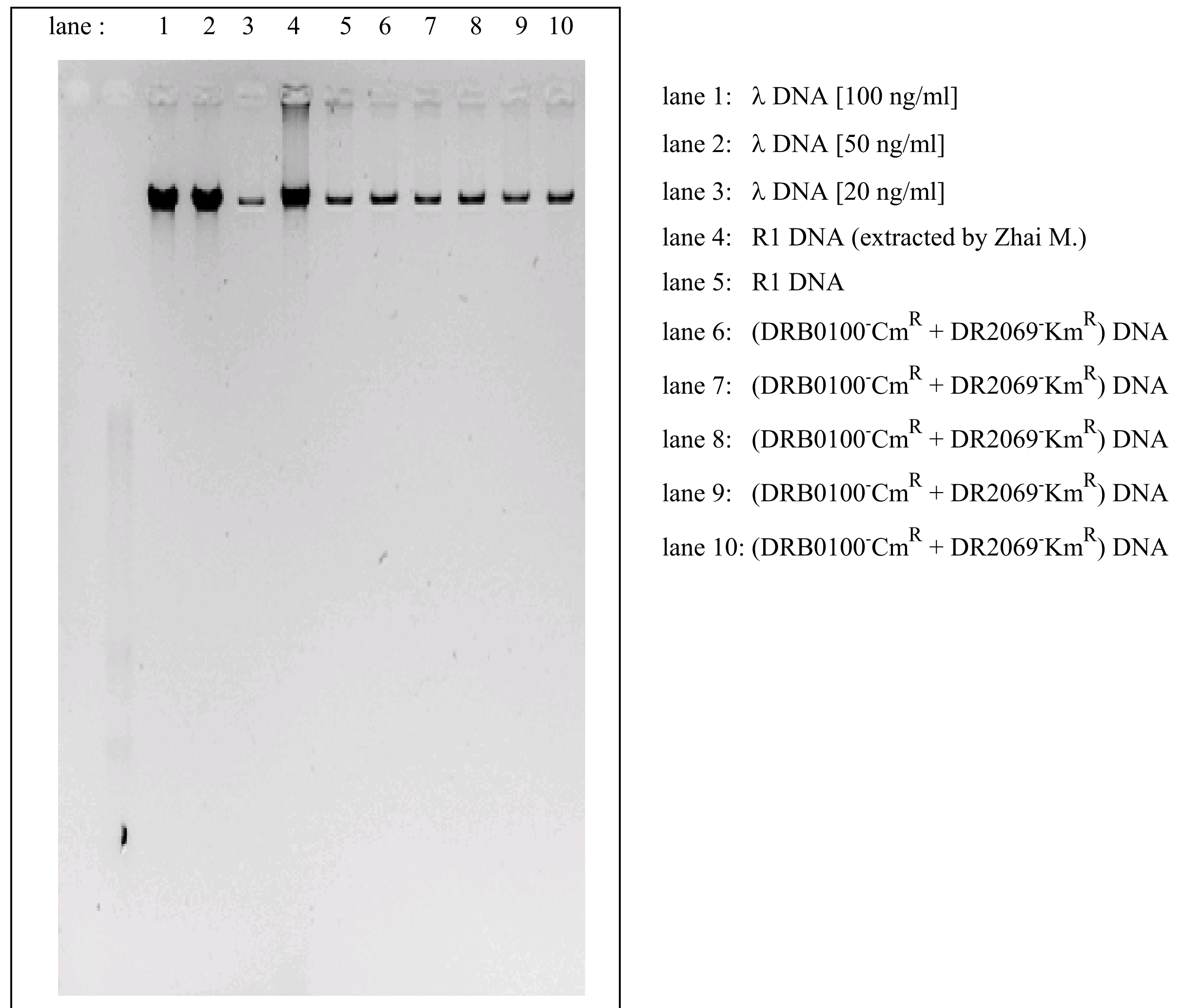
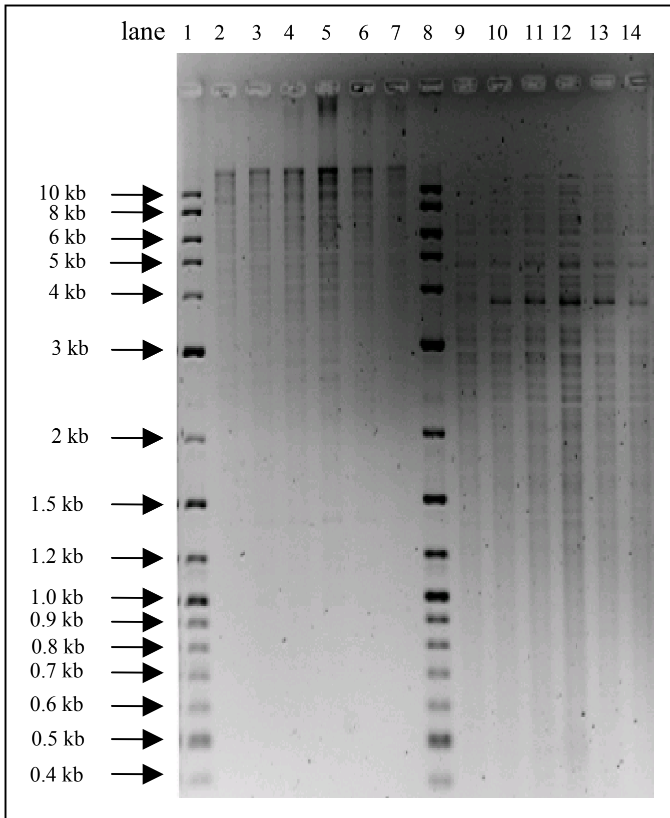


Figure 4.4. Agarose gel of DNA digested with *Alw*I/*Ava*I



lane 1: 2-log DNA ladder (New England Biolabs, MA)

lane 2: R1 DNA digested with *Alw*I

lane 3: MH46 DNA digested with *Alw*I

lane 4: MH47 DNA digested with *Alw*I

lane 5: MH48 DNA digested with *Alw*I

lane 6: MH49 DNA digested with *Alw*I

lane 7: MH50 DNA digested with *Alw*I

lane 8: 2-log DNA ladder (New England Biolabs, MA)

lane 9: R1 DNA digested with *Ava*I

lane 10: MH46 DNA digested with *Ava*I

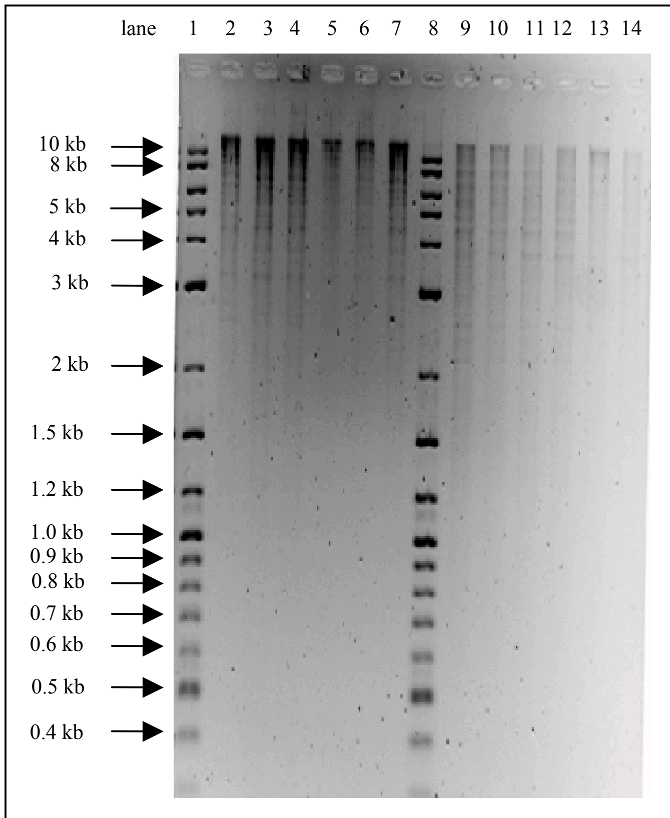
lane 11: MH47 DNA digested with *Ava*I

lane 12: MH48 DNA digested with *Ava*I

lane 13: MH49 DNA digested with *Ava*I

lane 14: MH50 DNA digested with *Ava*I

Figure 4.5. Agarose gel of DNA digested by *Bsa*I/*Hinc*II



lane 1: 2-log DNA ladder (New England Biolabs, MA)

lane 2: R1 DNA digested with *Bsa*I

lane 3: MH46 DNA digested with *Bsa*I

lane 4: MH47 DNA digested with *Bsa*I

lane 5: MH48 DNA digested with *Bsa*I

lane 6: MH49 DNA digested with *Bsa*I

lane 7: MH50 DNA digested with *Bsa*I

lane 8: 2-log DNA ladder (New England Biolabs, MA)

lane 9: R1 DNA digested with *Hinc*II

lane 10: MH46 DNA digested with *Hinc*II

lane 11: MH47 DNA digested with *Hinc*II

lane 12: MH48 DNA digested with *Hinc*II

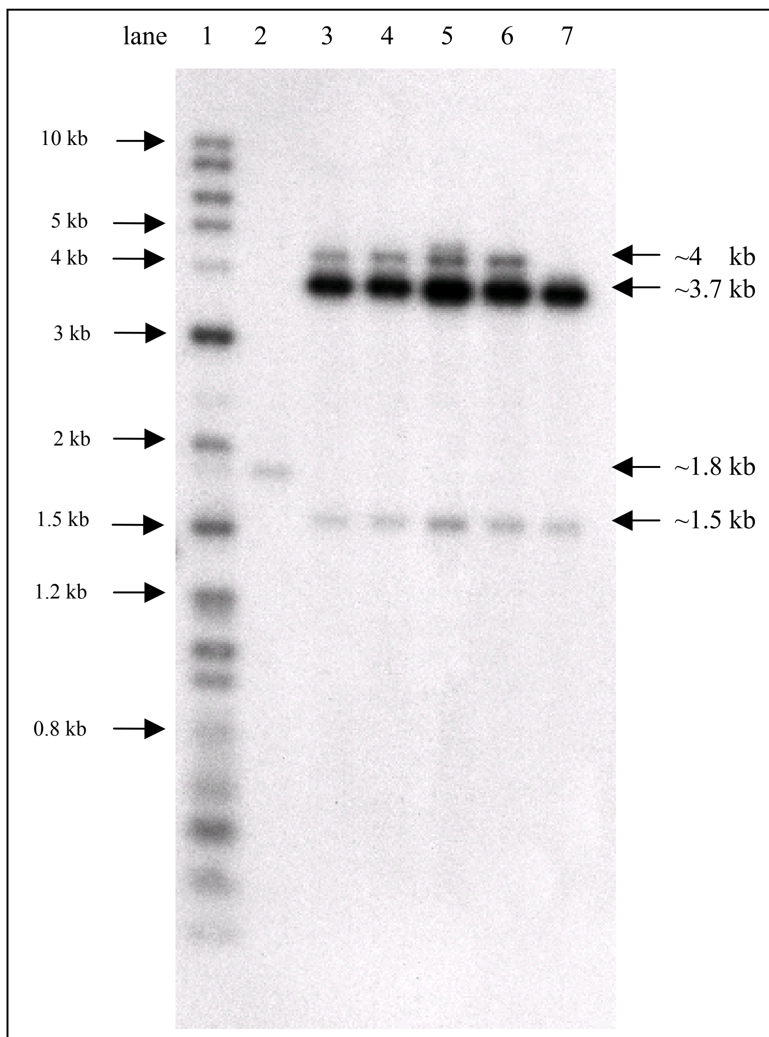
lane 13: MH49 DNA digested with *Hinc*II

lane 14: MH50 DNA digested with *Hinc*II

4.3.3. Southern Hybridization

Autoradiography showed that a $\alpha[^{32}P]$-dCTP labeled DRB0100-specific probe hybridized with three bands in each of the *Ava*I-digested mutant DNA samples (MH46, MH47, MH48, MH49, and MH50) (Fig. 4.6). The major band (~3.7 kbp) represents the amplified region of the duplication as shown in Fig. 4.7. The two fainter bands at positions 4.0 kb and 1.5 kb are derived from hybridization of the probe with the non-duplicated flanking regions of DRB100 (Fig 4.7). As expected, the wild-type strain shows a single band at ~1.8 kb. A similar genomic mapping for DR2069 showed that it did not contain bands predicted by a disruption of the DR2069 wild-type sequence with a pCR-based insertion vector (Figs. 4.8 & 4.9). Therefore, the disruption of DR2069 was lost during the construction of the [DRB0100^{-}Cm^{R} + DR2069^{-}Km^{R}] transformants. Only the wild-type DR2069 2.6 kb diagnostic band was present in strains MH46, MH47, MH48, MH49, and MH50 (Fig. 4.8), and a subsequent hybridization with a Km-probe showed that the insertion vector was absent (Fig 4.10). Km-probe was shown to be good for *aphA* detection on a separate membrane (data not shown).

Figure 4.6. Autoradiography: DRB0100-probe



lane 1: 2-log DNA ladder hybridized with ^{32}P labeled 2-log ladder probe

lane 2: R1 DNA digested with *Ava*I hybridized with ^{32}P labeled DRB0100 probe

lane 3: MH46 DNA digested with *Ava*I hybridized with ^{32}P labeled DRB0100 probe

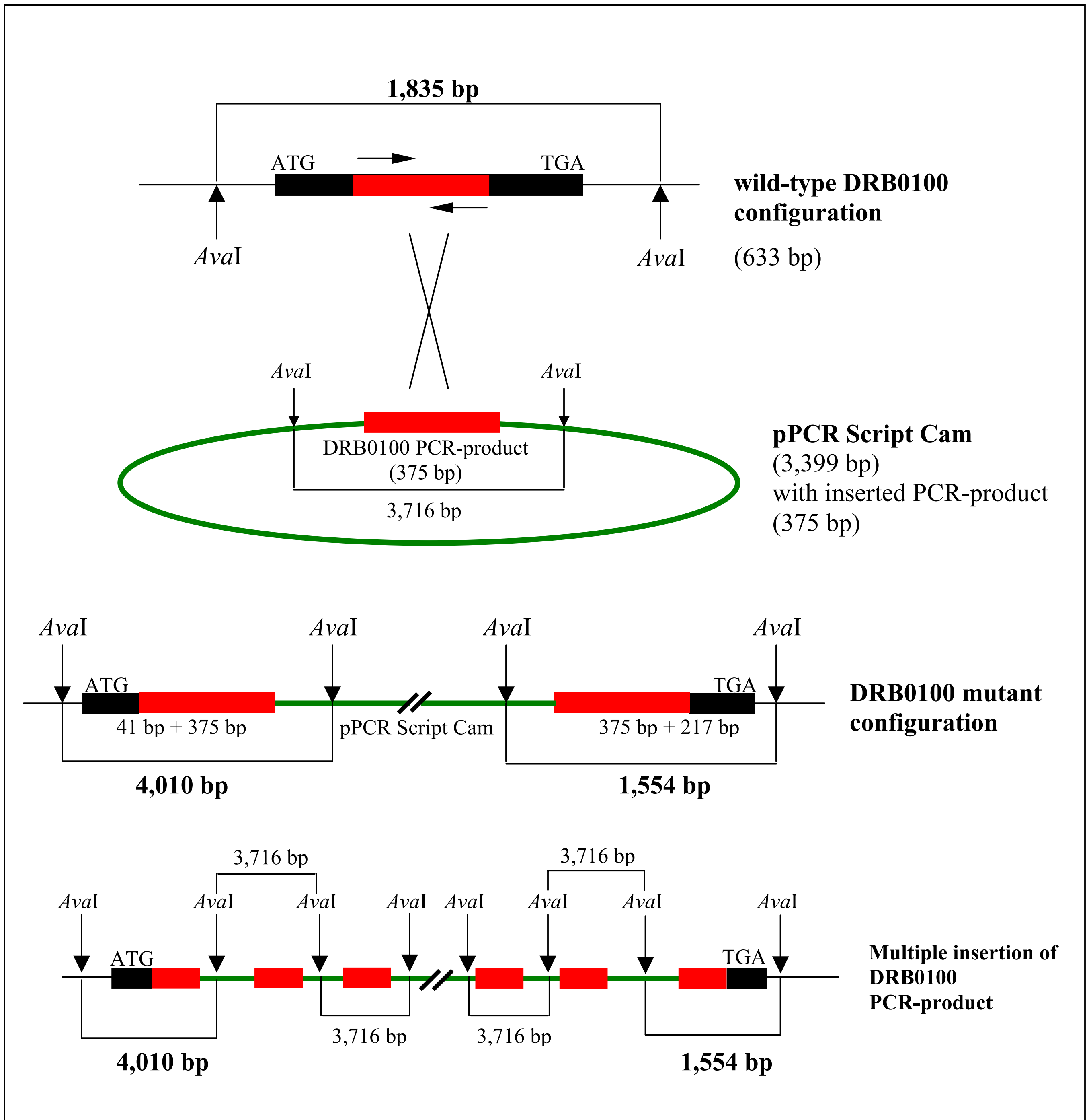
lane 4: MH47 DNA digested with *Ava*I hybridized with ^{32}P labeled DRB0100 probe

lane 5: MH48 DNA digested with *Ava*I hybridized with ^{32}P labeled DRB0100 probe

lane 6: MH49 DNA digested with *Ava*I hybridized with ^{32}P labeled DRB0100 probe

lane 7: MH50 DNA digested with *Ava*I hybridized with ^{32}P labeled DRB0100 probe

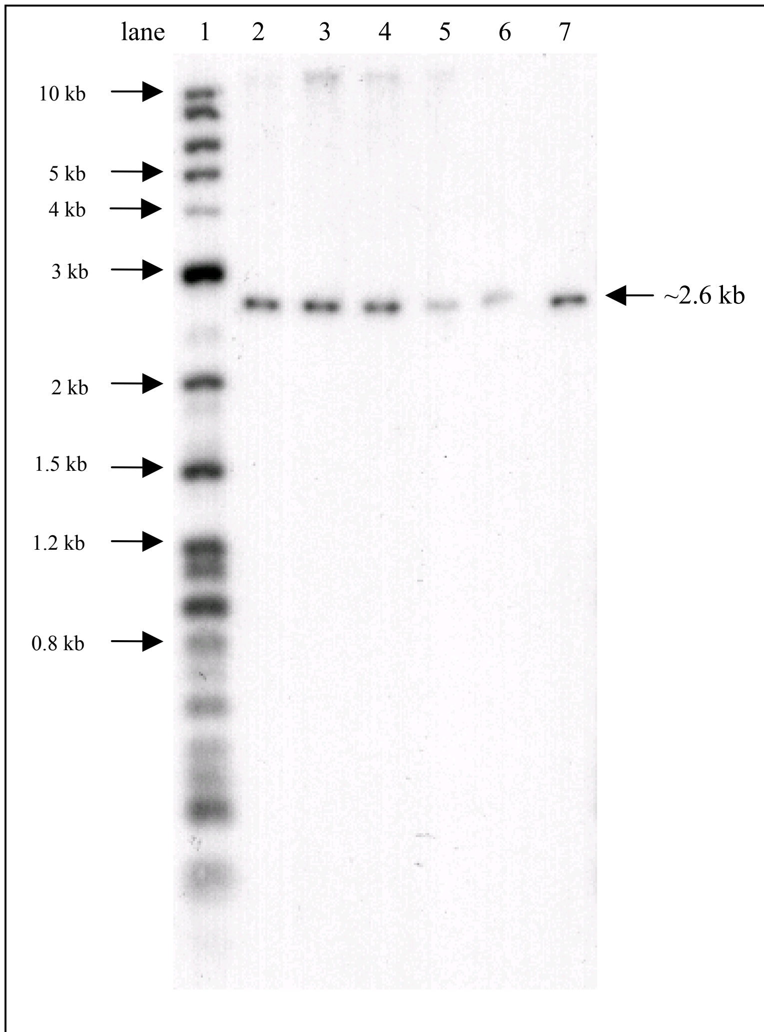
Figure 4.7. Map of insertion site in mutant DRB0100



Legend Fig. 4.7.

The wild-type gene DRB0100 (eukaryotic ligase) is represented by the black box with the start sequence ATG and the stop codon TGA, containing a sequence (red box) for which a diagnostic PCR probe sequence was generated. Horizontal arrows point to the location of the forward and reverse PCR primers used to generate the DRB0100 probe. Vertical arrows show the digestion sites for *Ava*I. Disruption vector pPCR Script Cam (illustrated in green) contains the DRB0100 PCR product (red box). The predicted length of bands in the mutant are 4,010 bp and 1,554 bp; for the wild-type sequence 1,835 bp. The 3,716 bp band is the amplified sequence.

Figure 4.8. Autoradiography: DR2069-probe



lane 1: 2-log DNA ladder hybridized with ^{32}P labeled 2-log ladder probe

lane 2: R1 DNA digested with *Bsa*I hybridized with ^{32}P labeled DR2069 probe

lane 3: MH46 DNA digested with *Bsa*I hybridized with ^{32}P labeled DR2069 probe

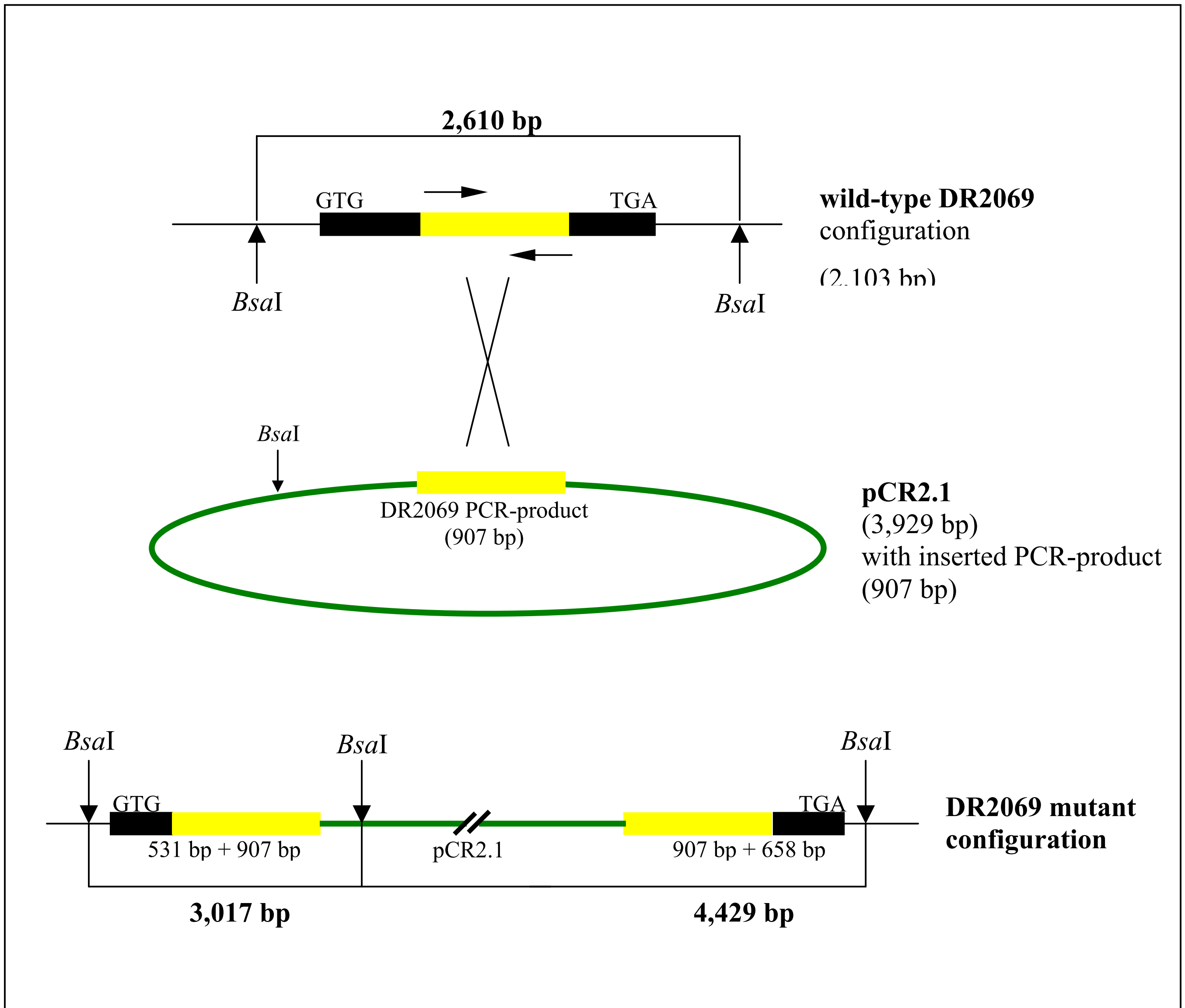
lane 4: MH47 DNA digested with *Bsa*I hybridized with ^{32}P labeled DR2069 probe

lane 5: MH48 DNA digested with *Bsa*I hybridized with ^{32}P labeled DR2069 probe

lane 6: MH49 DNA digested with *Bsa*I hybridized with ^{32}P labeled DR2069 probe

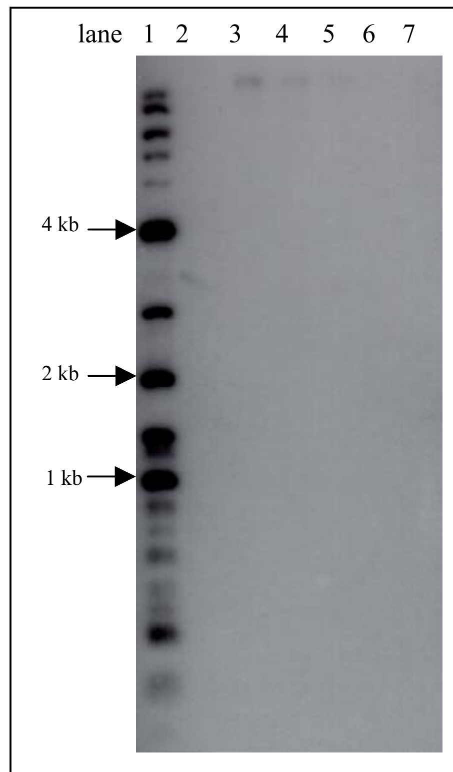
lane 7: MH50 DNA digested with *Bsa*I hybridized with ^{32}P labeled DR2069 probe

Figure 4.9. Map of insertion site in mutant DR2069



Legend Fig. 4.9.
The wild-type gene DR2069 (bacterial ligase) is represented by the black box with the start sequence GTG and the stop codon TGA, containing a sequence (yellow box) for which a homologue PCR product was generated. Horizontal arrows point to the location of the forward and reverse PCR primers used to generate the DR2069 probe. Vertical arrows show the digestion sites for *Bsa*I. Disruption vector pCR2.1 (illustrated in green) contains the PCR product (yellow box). The predicted length of bands in the mutant are 3,017 bp and 4,429 bp; for the wild-type sequence 2,610 bp.

Figure 4.10. Autoradiography: Km^R-probe



lane 1: 2-log DNA ladder (New England Biolabs, MA) with ^{32}P labeled 2-log ladder probe

lane 2: R1 DNA digested with *Bsa*I

lane 3: MH46 DNA digested with *Bsa*I

lane 4: MH47 DNA digested with *Bsa*I

lane 5: MH48 DNA digested with *Bsa*I

lane 6: MH49 DNA digested with *Bsa*I

lane 7: MH50 DNA digested with *Bsa*I

4.4. Discussion

Annotation of the *D. radiodurans* genome predicted the presence of two distinct DNA ligases (DR2069 and DRB0100). DR2069 and DRB0100 were previously targeted for disruption by insertional inactivation, which resulted in heterozygous DR2069 and homozygous DRB0100 single mutants. Both single mutants showed wild-type radiation resistance to chronic or acute doses of radiation (Table 2.1). The double mutant constructions (DR2069^{-}Km^{R} + DRB0100^{-}Cm^{R}) were examined for their radiation resistance phenotype (Chapter 3). Three clones (MH48-MH50) were shown to be more sensitive to ionizing radiation than either of the parental strains (Fig. 3.3; Table 3.6). Prospective double mutants were subjected to Southern analysis using diagnostic radio-labeled probes (Chapter 4) to determine the structure of ORFs DR2069 and DRB0100. Southern blotting of the Km^{R}-Cm^{R} transformants confirmed the disruption of DRB0100 (Figs. 4.6 & 4.7), but not DR2069 (Figs. 4.8 & 4.9); DR2069 had a wild-type configuration without any evidence of inserted plasmid sequences. As such, the radiation sensitive phenotype of the Km^{R}-Cm^{R} transformants was likely due to the introduction of an unknown second mutation in combination with DRB0100^{-}. Since the Km^{R}-Cm^{R} transformants only showed *E. coli* plasmid sequences at locus DRB0100, this supports that the secondary mutation(s) were spontaneous and not due to the insertion of the transforming Km^{R} vector into another region of the genome.

This laboratory has previously shown that spontaneous Km resistant mutants of *D. radiodurans* are frequently more sensitive to radiation than wild-type (E. Gaidamakova, unpublished). Genotypic analyses of these spontaneous Km^{R} mutants showed that the gene DR0066, which is predicted to confer Km resistance, exists in multiple copies, *i.e.*, present within a genomic amplification. The relationship between the genomic amplification and Km resistance is currently being examined, with emphasis on the role of DR0070 (Table 2.1) within this genomic region. To determine if DR0066 is involved in the antibiotic resistance of strains MH46-50, further genomic characterization would be required as described in Chapter 4. Since several independent attempts to disrupt ORF DR2069 resulted in heterozygous mutants (Matrosova V. Y., *et al*., 2003), it is possible that DR2069 is an essential gene and cannot be eliminated from the genome.

4.5. Conclusions

1. DR2069^{-}Km^{R} + DRB0100^{-}Cm^{R} double mutant construction did not yield the predicted genotype in radiation sensitive derivatives. Whereas DRB0100 was disrupted, DR2069 was not. This result is consistent with a secondary, as yet uncharacterized, mutation in the constructs, which renders the cells sensitive to acute radiation.

2. DR2069 likely is an essential gene.

Chapter 5: General Discussion

The radiation resistance mechanisms of *D. radiodurans* have been the subject of continuous research since its isolation in 1956 (Anderson A. W., *et al*., 1956, Andersson A. M., *et al*., 1999). Perhaps of prime importance in driving this research in recent years has been the development of *D. radiodurans* for bioremediation of highly radioactive waste sites leftover from the Cold War (Brim H., *et al*., 2000). Two of the most prevalent classes of radioactive waste constituents are heavy metals (*e.g*., Hg, Cr, & Pb) and toxic aromatic compounds (*e.g*., toluene), and *D. radiodurans* has been developed for treatment of combinations of both classes. For example, the most recently engineered *D. radiodurans* strain is able to use energy derived from toluene to facilitate chromate reduction in radioactive environments (Brim H., *et al*., 2003, In Press).

Although it is believed that the resistance mechanisms of *D. radiodurans* stem from efficient DNA repair processes (Daly M. J., *et al*., 1994 a; Daly M. J., *et al*., 1994 b; Daly M. J., Minton K. W., 1997), the genes encoding those functions remain poorly understood. Currently, only a few genes known to participate directly in the extreme resistance phenotype have been identified, including *recA*, *polA*, *irrE*, and *uvrA* (Gutman P. D., *et al*., 1993; Gutman P. D., *et al*., 1994; Funayama T., *et al*., 1999; Earl A. M., *et al*., 2002 a; Hua Y., *et al*., 2003). The principal aim of this thesis was to characterize three novel genes predicted to be involved in recovering from ionizing radiation by examining their effect on resistance to acute and chronic radiation, desiccation and temperature. The mutants under investigation contained disruptions in one or more genes identified by a combination of comparative genomics and expression profiling (Makarova K. S., *et al*., 2001, Liu Y., *et al*., 2003).

Previous reports suggest that the extreme radiation resistance phenotype of *D. radiodurans* may be the result of organisms that evolved in genotoxic environments. While naturally radioactive environments on Earth are rare, high levels of DNA damage can be inflicted by a variety of other conditions. For example, organisms subjected to environments exposed to cycles of desiccation and hydration are vulnerable to elevated oxidative stress levels generated by metabolic processes (Mattimore V., Battista J. R.,

1996; Makarova K. S., *et al*., 2001, Walters C., *et al*., 2002). The expression levels of several hundred genes of *D. radiodurans* recently were shown to be increased following exposure of cells to 15 kGy (Liu Y., *et al*., 2003). For those genes that showed elevated expression levels, computational approaches were used to prioritize a list of ~100 genes targeted for disruption and further characterization (Gaidamakova E. K., unpublished). Work presented here examined the role of three of those genes (DR0070, DR0105, and DR2339) in the recovery from DNA damage. Specifically, knockout mutants were examined for their ability to recover from exposure to desiccation (Chapter 2), growth at 37˚C (Chapter 2), and for their effect on resistance to chronic and acute exposures to γ radiation (Table 2.1). Several conclusions based on this work support that the variety of resistance phenotypes of *D. radiodurans* is diverse and based on different mechanisms. For example, the homozygous DR0070 mutant is sensitive to acute radiation, but not chronic radiation or desiccation (Table 2.1 & 2.4). This suggests that the mechanisms involved in acute radiation and those involved in chronic radiation and desiccation resistance are different. This finding is consistent with a recent report by Battista's group (Battista J. R., *et al*., 2001) that showed that disruption of predicted desiccation genes of *D. radiodurans* did not affect its resistance to acute radiation, and illustrates the difficulty in assigning function to uncharacterized genes. Mutant DR0105 was predicted to be involved in the desiccation resistance phenotype (Makarova K. S., *et al*., 2001), but was found to be resistant to desiccation, acute and chronic radiation, and could grow at 37˚C (Table 2.1; Figs. 2.4 & 2.12). In contrast, mutant DR2339 was sensitive to growth at 37˚C (Fig. 2.5) and under chronic radiation, but not to acute radiation (Table 2.1). This supports that DR2339 may be involved in oxidative stress suppression since the level of ROS are likely increased during metabolism at elevated temperatures or under chronic radiation (Venkateswaran A., *et al*., 2000; Harada K., *et al*., 2003).

Sequencing and annotation of the deinococcal genome showed the presence of two genes encoding distinct DNA ligases (White O., *et al*., 1999; Makarova K. S., *et al*., 2001). Whereas disruption of a NAD-dependant DNA ligase in *E. coli* resulted in strains sensitive to elevated temperature and ultraviolet radiation (Nagata T., Horiuchi T., 1974; Dermody J. J. *et al*., 1979), it was not possible to fully characterize the role of DR2069 in *D. radiodurans* since a homozygous disruption of ORF DR2069 could not be

constructed. The issue of persistent heterozygosity illustrates some of the challenges in mutant construction and assigning functions to genes. For example, based on the induction of DR2069 following irradiation (Liu Y., *et al*., 2003) it seems likely that it is involved in recovery, but because it likely is an essential gene, any role it has in recovery will be difficult to confirm. This applies to any gene that is essential for *D. radiodurans* growth. An alternative strategy to isolate the role of essential genes in the resistance phenotypes would be to use engineered point mutations or, possibly, bacterial gene silencing techniques based on RNA/DNA hybrid molecules now under development (Christian A. T., unpublished data). Such approaches might provide a way to modulate the expression of single genes and correlate the level of gene product with changes in resistance. Notwithstanding these developing possibilities, mutant construction in combination with physiological assays remains the most established experimental approach to determine gene function. Collectively, results presented in this thesis underscore the complexity of the resistance phenotypes of *D. radiodurans* and the need to further characterize the genetics of survival using a systematic approach based on single and double mutant analyses.

Bibliography of Literature

Adams D. G., 2000. Cyanobacterial phylogeny and development: questions and challenges, in: Brun Y. V., Shimkets L. J. (eds.). Prokaryotic development, ASM Press, Washington, DC, pp. 51-81.

Anderson A. W., Nordan H. C., Cain R. F., Parrish G., Duggan D., 1956. Studies on a radio-resistant *micrococcus*. I. Isolation, morphology, cultural characteristics and resistance to gamma radiation. Food Technol. 10:575-578.

Andersson A. M., Weiss N., Rainey F., Salkinoja-Salonen M. S., 1999. Dust-borne bacteria in animal sheds, schools and children's day care centres. J. Appl. Microbiol. 86:622-634.

Bale M. J., Bennett P. M., Beringer J. E., Hinton M., 1993. The survival of bacteria exposed to desiccation on surfaces associated with farm buildings. J. Appl. Bacteriol. 75:519-528.

Battista J. R., Earl A. M., Park M. J., 1999. Why is *Deinococcus radiodurans* so resistant to ionizing radiation? Trends Microbiol. 7:362-365.

Battista J. R., Park M. J., McLemore A. E., 2001. Inactivation of two homologues of proteins presumed to be involved in the desiccation tolerance of plants sensitizes *Deinococcus radiodurans* R1 to desiccation. Cryobiology 43:133-139.

Baumeister W., Kübler O., 1978. Topographic study of the cell surface of *Micrococcus radiodurans.* Proc. Natl. Acad. Sci. USA 75:5525-5528.

Boylen C. W., 1973. Survival of *Arthrobacter crystallopoietes* during prolonged periods of extreme desiccation. J. Bacteriol. 113:33-37.

Brim H., McFarlan S. C., Fredrickson J. K., Minton K. W., Zhai M., Wackett L. P., Daly M. J., 2000. Engineering *Deinococcus radiodurans* for metal remediation in radioactive mixed waste environments. Nat. Biotechnology 18:85-90.

Brim H., Osborne J. P., Zhai M., Konstandarithes H. M., Fredrickson J. K., Wackett L. P., Daly M. J., 2003. Appl. Environ. Microbiol., in press.

Brim H., Venkateswaran A., Kostandarithes H. M., Fredrickson J. K., Daly M. J., 2003. Engineering *Deinococcus geothermalis* for bioremediation of high-temperature radioactive waste environments. Appl. Environ. Microbiol. 69:4575-4582.

Brooks B. W., Murray R. G. E., 1981. Nomenclature for "*Micrococcus radiodurans*" and other radiation-resistant cocci: *Deinococcaceae* fam. nov. and *Deinococcus* gen. nov. including five species. Int. J. Syst. Bacteriol. 31:353-360.

Brooks B. W., Murray R. G. E., Johnson J. L., Stackebrandt E., Woese C. R., Fox G. E., 1980. Red-pigmented micrococci: a basis for taxonomy. Int. J. Syst. Bacteriol. 30:627-646.

Bruce A. K., 1964. Extraction of the radioresistant factor of *Micrococcus radiodurans*. Radiation Research 22:155-164.

Christensen E. A., 1964. Radiation resistance of *Enterococci* dried in air. Acta. Pathol. Microbiol. Scand. 61:483-486.

Christensen E. A., Kristensen H., 1981. Radiation-resistance of micro-organisms from air in clean premises. Acta. Pathol. Microbiol. Scand. 89:293-301.

Crowe J. H., Crowe L. M., Carpenter J. E., Petrelski S., Hoekstra F. A., De Araujo P., Panek A. D., 1997. Anhydrobiosis: cellular adaptation to extreme dehydration, in:

Dantzler W. H. (ed.). Handbook of physiology, Vol. II, Oxford University Press, Oxford, pp. 1445-1478.
Crowe L. M., Crowe J. H., 1992. Anhydrobiosis: a strategy for survival. Adv. Space Res. 12:239-247.
Daly M. J., 2000. Engineering radiation-resistant bacteria for environmental biotechnology. Curr. Opin. Biotechnol. 11:280-285.
Daly M. J., Ling O., Minton K. W., 1994 a. Interplasmidic recombination following irradiation of the radioresistant bacterium *Deinococcus radiodurans*. J. Bacteriol. 176:7506-7515.
Daly M. J., Minton K. W., 1995 a. Interchromosomal recombination in the extremely radioresistant bacterium *Deinococcus radiodurans*. J. Bacteriol. 177:5495-5505.
Daly M. J., Minton K. W., 1995 b. Resistance to radiation. Science 270:1318.
Daly M. J., Minton K. W., 1996. An alternative pathway of recombination of chromosomal fragments precedes *recA*-dependent recombination in the radioresistant bacterium *Deinococcus radiodurans*. J. Bacteriol. 178:4461-4471.
Daly M. J., Minton K. W., 1997. Recombination between a resident plasmid and the chromosome following irradiation of the radioresistant bacterium *Deinococcus radiodurans.* Gene 187:225-229.
Daly M. J., Ouyang L., Fuchs P., Minton K. W., 1994 b. In vivo damage and *recA*-dependent repair of plasmid and chromosomal DNA in the radiation-resistant bacterium *Deinococcus radiodurans*. J. Bacteriol. 176:3508-3517.
Davis N. S., Silverman G. J., Mausurosky E. B., 1963. Radiation resistant, pigmented coccus isolated from haddock tissue. J. Bacteriol. 86:294-298.
Dermody J. J., Robinson G. T., Sternglanz R., 1979; Conditional-lethal deoxyribonucleic acid ligase mutant of *Escherichia coli*. J. Bacteriol. 139:701-704.
Doherty A. J., Suh S. W., 2000. Structural and mechanistic conservation in DNA ligases. Nucleic Acids Res. 28:4051-4058.
Earl A. M., Mohundro M. M., Mian I. S., Battista J. R., 2002 a. The IrrE protein of *Deinococcus radiodurans* R1 is a novel regulator of *recA* expression. J. Bacteriol. 184:6216-6224.
Earl A. M., Rankin S. K., Kim K. P., Lamendola O. N., Battista J. R., 2002 b. Genetic evidence that the *uvsE* gene product of *Deinococcus radiodurans* R1 is a UV damage endonuclease. J. Bacteriol. 184:1003-1009.
Embley T. M., O'Donnell A. G., Wait R., Rostron J., 1987. Lipid and cell wall amino acid composition in the classification of members of the genus *Deinococcus*. Syst. Appl. Microbiol. 10:20-27.
Ferreira A. C., Nobre M. F., Rainey F. A., Silva M. T., Wait R., Burghardt J., Chung A. P., Da Costa M. S., 1997. *Deinococcus geothermalis* sp. nov. and *Deinococcus murrayi* sp. nov., two extremely radiation-resistant and slightly thermophilic species from hot springs. Int. J. Syst. Bacteriol. 47:939-947.
Fredrickson J. K., Kostandarithes H. M., Li S. W., Plymale A. E., Daly M. J., 2000. Reduction of Fe(III), Cr(VI), U(VI), and Tc(VII) by *Deinococcus radiodurans* R1. Appl. Environ. Microbiol. 66:2006-2011.
Funayama T., Narumi I., Kikuchi M., Kitayama S., Watanabe H., Yamamoto K., 1999. Identification and disruption analysis of the *recN* gene in the extremely radioresistant bacterium *Deinococcus radiodurans*. Mutat. Res. 435:151-161.

Gaidamakova E. K., Matrosova V. Y., Zhai M., Hess M., Liu Y., Omelchenko M., Makarova K. S., Zhou J., Daly M. J., 2003. Predicted genes important for the extreme radiation resistance of *Deinococcus radiodurans*. 12th International Congress of Radiation Research. Brisbane, Queensland Australia.

Grant I. R., Patterson M. F., 1989. A novel radiation resistant *Deinobacter* sp. isolated from irradiated pork. Lett. Appl. Microbiol. 8:21-24.

Gutman P. D., Carroll J. D., Masters C. I., Minton K. W., 1994. Sequencing, targeted mutagenesis and expression of a *recA* gene required for the extreme radioresistance of *Deinococcus radiodurans*. Gene 141:31-37.

Gutman P. D., Fuchs P., Ouyang L., Minton K. W., 1993. Identification, sequencing, and targeted mutagenesis of a DNA polymerase gene required for the extreme radioresistance of *Deinococcus radiodurans*. J. Bacteriol. 175:3581-3590.

Gutteridge J. M. C., Halliwell B., 1999. Free Radicals in Biology and Medicine. Third edition. Oxford University Press, New York.

Hanada K., Yamashita T., Shobuike Y., Ikeda H., 2001. Role of DnaB helicase in UV-induced illegitimate recombination in *Escherichia coli*. J. Bacteriol. 183:4964-4969.

Hansen M. T., 1978. Multiplicity of genome equivalents in the radiation-resistant bacterium *Micrococcus radiodurans*. J. Bacteriol. 134:71-75.

Harada K., Ando M., Fujimura-Ito T., Kasahara-Imamura M., Sako Y., Uchida A., Ishida Y., Kadota H., Ohnishi T., Moseley B. E., 2003. Mechanism of thermotolerance induction by split-dose hyperthermia in *Deinococcus radiodurans* DNA repair deficient mutants. Int. J. Mol. Med. 12:741-747.

Harsojo, Kitayama S., Matsuyama A., 1981. Genome multiplicity and radiation resistance in *Micrococcus radiodurans*. J. Biochem. 90:877-880.

Hensel R., Demharter W., Kandeler O., Kroppenstedt R. M., Stackebrandt E., 1986. Chemotaxonomic and molecular-genetic studies of the genus *Thermus*: Evidence for a phylogenetic relationship of *Thermus aquaticus* and *Thermus ruber* to the genus *Deinococcus*. Int. J. Syst. Bacteriol. 36: 444-453.

Hua Y., Narumi I., Gao G., Tian B., Satoh K., Kitayama S., Shen B., 2003. *pprI*: a general switch responsible for extreme radioresistance of *Deinococcus radiodurans*. Biochem. Biophys. Res. Commun. 306:354-360.

Ito H., Watanabe H., Takeshia M., Iizuka H., 1983. Isolation and identification of radiation-resistant cocci belonging to the genus *Deinococcus* from sewage sludges and animal feeds. Agric. Biol. Chem. 47: 1239-1247.

Jonsson K. I., Rebecchi L., 2002. Experimentally induced anhydrobiosis in the tardigrade *Richtersius coronifer*: phenotypic factors affecting survival. J. Exp. Zool. 293:578-584.

Kitayama S., Matsuyama A., 1971. Mechanism for radiation lethality in *M. radiodurans.* Int. J. Radiat. Biol. Relat. Stud. Phys. Chem. Med. 19:13-9.

Krasin F., Hutchinson F., 1977. Repair of DNA double-strand breaks in *Escherichia coli*, which requires *recA* function and the presence of a duplicate genome. J. Mol. Biol. 116:81-98.

Kristensen H., Christensen E. A., 1981. Radiation-resistant micro-organisms isolated from textiles. Acta. Pathol. Microbiol. Scand. 89:303-309.

Kuzminov A., 1999. Recombinational repair of DNA damage in *Escherichia coli* and bacteriophage lambda. Microbiol. Mol. Biol. Rev. 63:751-813.

Lange C. C., Wackett L. P., Minton K. W., Daly M. J., 1998. Engineering a recombinant *Deinococcus radiodurans* for organopollutant degradation in radioactive mixed waste environments. Nat. Biotechnol. 16:929-933.

Levin-Zaidman S., Englander J., Shimoni E., Sharma A. K., Minton K. W., Minsky A., 2003. Ringlike structure of the *Deinococcus radiodurans* genome: a key to radioresistance? Science 299:254-256.

Lin J., Qi R., Aston C., Jing J., Anantharaman T. S., Mishra B., White O., Daly M. J., Minton K. W., Venter J. C., Schwartz D. C., 1999. Whole-genome shotgun optical mapping of *Deinococcus radiodurans.* Science 285:1558-1562.

Linders L. J., Wolkers W. F., Hoekstra F. A., van't Riet K., 1997. Effect of added carbohydrates on membrane phase behavior and survival of dried *Lactobacillus plantarum*. Cryobiology 35:31-40.

Lipton M. S., Pasa-Tolic' L., Anderson G. A., Anderson D. J., Auberry D. L., Battista J. R., Daly M. J., Fredrickson J., Hixson K. K., Kostandarithes H., Masselon C., Markillie L. M., Moore R. J., Romine M. F., Shen Y., Stritmatter E., Tolic' N., Udseth H. R., Venkateswaran A., Wong K. K., Zhao R., Smith R. D., 2002. Global analysis of the *Deinococcus radiodurans* proteome by using accurate mass tags. Proc. Natl. Acad. Sci. USA 99:11049-11054.

Liu Y., Zhou J., Omelchenko M. V., Beliaev A. S., Venkateswaran A., Stair J., Wu L., Thompson D. K., Xu D., Rogozin I. B., Gaidamakova E. K., Zhai M., Makarova K. S. , Koonin E. V., Daly M. J., 2003. Transcriptome dynamics of *Deinococcus radiodurans* recovering from ionizing radiation. Proc. Natl. Acad. Sci. USA 100:4191-4196.

Majumdar S., Chandra A. K., 1985. UV-repair and mutagenesis in *Azotobacter vinelandii*. Repair of UV-induced damages. Zentralbl. Mikrobiol. 140:247-254.

Makarova K. S., Aravind L., Wolf Y. I., Tatusov R. L., Minton K. W., Koonin E. V., Daly M. J., 2001. Genome of the extremely radiation-resistant bacterium *Deinococcus radiodurans* viewed from the perspective of comparative genomics. Microbiol. Mol. Biol. Rev. 65:44-79. Review.

Markillie L. M., Varnum S. M., Hradecky P., Wong K. K., 1999. Targeted mutagenesis by duplication insertion in the radioresistant bacterium *Deinococcus radiodurans*: Radiation sensitivities of catalase (*katA*) and superoxide dismutase (*sodA*) mutants. J. Bacteriol. 181: 666-669.

Matrosova V. Y., Omelchenko M. V., Venkateswaran A., Zhai M., Hess M., Gaidamakova E. K., Makarova K. S., Zhou J., Daly M. J., 2003. Analysis of Novel *Deinococcus radiodurans* mutants following whole genome transcriptome analysis. New Horizons in Genomics. U. S. Department of Energy Joint Genome Institute, Santa Fe, New Mexico.

Mattimore V., Battista J. R., 1996. Radioresistance of *Deinococcus radiodurans*: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. J. Bacteriol. 178:633-637.

Maxcy R. B., Rowley D. B., 1978. Radiation-resistant vegetative bacteria in a proposed system of radappertization of meats. In food preservation by irradiation. Vienna: International Atomic Energy Agency.

Michel B., Ehrlich S. D., Uzest M., 1997. DNA double-strand breaks caused by replication arrest. EMBO J. 16:430-438.

Minton K. W., 1994. DNA repair in the extremely radioresistant bacterium *Deinococcus radiodurans*. Mol. Microbiol. 13:9-15.

Minton K. W., 1996. Repair of ionizing-radiation damage in the radiation resistant bacterium *Deinococcus radiodurans*. Mutat. Res. 363:1-7. Review.

Moseley B. E. B., 1983. Photobiology and radiobiology of *Micrococcus (Deinococcus) radiodurans*. Photochem. Photobiol. Rev. 7:223-275.

Moseley B. E., Evans D. M., 1983. Isolation and properties of strains of *Micrococcus (Deinococcus) radiodurans* unable to excise ultraviolet light-induced pyrimidine dimers from DNA: evidence for two excision pathways. J. Gen. Microbiol. 129 (Pt 8):2437-2445.

Murray R. G. E., 1986. Genus 1. *Deinococcus* Brooks and Murray 1981, in: Sneath P. H. A., Mair N. S., Sharpe M. E., Holt J. G. (eds.). Bergey's Manual of Systematic Bacteriology 2:1035-1043. Baltimore: Williams & Wilkins.

Murray R. G. E., 1992. The family *Deinococcaceae*. In: Ballows A., Truper H. G., Dworkin M., Harder W., Schleifer K. H. (eds.). The Prokaryotes 4:3732-44. New York, Springer-Verlag.

Murray, R. G. E., Hall, M., Thompson, B. G., 1983. Cell division in *Deinococcus radiodurans* and a method for displaying septa. Can. J. Microbiol. 29:1412-1423.

Nagata T., Horiuchi T., 1974; An amber *dna* mutant of *Escherichia coli* K12 affecting DNA ligase. J. Mol. Biol. 87:369-373.

Nelson K. E., Clayton R. A., Gill S. R., Gwinn M. L., Dodson R. J., Haft D. H., Hickey E. K., Peterson J. D., Nelson W. C., Ketchum K. A., McDonald L., Utterback T. R., Malek J. A., Linher K. D., Garrett M. M., Stewart A. M., Cotton M. D., Pratt M. S., Phillips C. A., Richardson D., Heidelberg J., Sutton G. G., Fleischmann R. D., Eisen J. A., Fraser C. M., 1999. Evidence for lateral gene transfer between archaea and bacteria from genome sequence of *Thermotoga maritima*. Nature 399:323-329.

Nicholson W. L., Munakata N., Horneck G., Melosh H. J., Setlow P., 2000. Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. Microbiol. Mol. Biol. Rev. 64:548-572.

Punita S. J., Reddy M. A., Das H. K., 1989. Multiple chromosomes of *Azotobacter vinelandii*. J. Bacteriol. 171:3133-3138.

Rainey F. A., Nobre M. F., Schumann P., Stackebrandt E., Da Costa M. S., 1997. Phylogenetic diversity of the *Deinococci* as determined by 16S ribosomal DNA sequence comparison. Int. J. Syst. Bacteriol. 47:510-514.

Saffary R., Nandakumar R., Spencer D., Robb F. T., Davila J. M., Swartz M., Ofman L., Thomas R. J., DiRuggiero J., 2002. Microbial survival of space vacuum and extreme ultraviolet irradiation: strain isolation and analysis during a rocket flight. FEMS Microbiol. Lett. 215:163-168.

Smith M. D., Masters C. I., Moseley B. E., 1992. Molecular biology of radiation resistant bacteria, pp. 258-280, in: Herbert R. A. and Sharp R. J. (eds.). Molecular biology and biotechnology of extremophiles. Chapman & Hall, New York.

Sweet D. M., Moseley B. E., 1974. Accurate repair of ultraviolet-induced damage in *Micrococcus radiodurans.* Mutat. Res. 23:311-318.

Thornley M. J., Horne R. W., Glauert A. M., 1965. The fine structure of *Micrococcus radiodurans*. Arch. Microbiol. 51:267-289.

United Nations Scientific Committee on the Effects of Atomic Radiation, 2000. Report to the General Assembly. United Nations Publications 2:4.
United Nations Scientific Committee on the Effects of Atomic Radiation. 1982. Report to the General Assembly. United Nations Publication.
Venkateswaran A., McFarlan S. C., Ghosal D., Minton K. W., Vasilenko A., Makarova K., Wackett L. P., Daly M. J., 2000. Physiologic determinants of radiation resistance in *Deinococcus radiodurans*. Appl. Environ. Microbiol. 66:2620-2626.
Wang P., Schellhorn H. E., 1995. Induction of resistance to hydrogen peroxide and radiation in *Deinococcus radiodurans*. Can. J. Microbiol. 41:170-176.
Walters C., Farrant F. M., Pammenter N. W., Berjak P., 2002. Desiccation stress and damage. In: Black M., Pritchard (eds.). Desiccation and survival in plants. CABI Publishing, NY.
Weisburg W. G., Giovannoni S. J., Woese C. R., 1989. The *Deinococcus-Thermus* phylum and the effect of rRNA composition on phylogenetic tree construction. Syst. Appl. Microbiol. 11:128-134.
White O., Eisen J. A., Heidelberg J. F., Hickey E. K., Peterson J. D., Dodson R. J., Haft D. H., Gwinn M. L., Nelson W. C., Richardson D. L., Moffat K. S., Qin H., Jiang L., Pamphile W., Crosby M., Shen M., Vamathevan J. J., Lam P., McDonald L., Utterback T., Zalewski C., Makarova K. S., Aravind L., Daly M. J., Minton K. W., Fleischmann R. D., Ketchum K. A., Nelson K. E., Salzberg S. L., Smith H. O., Venter J. C., Fraser C. M. 1999. Genome sequence of the radioresistant bacterium *Deinococcus radiodurans* R1. Science 286:1571-1577.
Work E., Griffith H., 1968. Morphology and chemistry of cell walls of *Micrococcus radiodurans*. J. Bacteriol. 95:641-657.