IDENTIFICATION AND CHARACTERIZATION OF *UVRA*, A DNA REPAIR GENE OF *DEINOCOCCUS RADIODURANS*

1996

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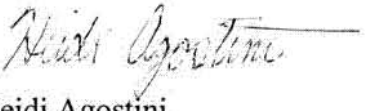

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ABSTRACT

Title of Dissertation:

Identification and Characterization of *uvrA*, a DNA Repair Gene of *Deinococcus radiodurans*.

Heidi J. Agostini

Doctor of Philosophy, 1996

Dissertation directed by:

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Gram positive bacteria of the genus Deinococcus possess extraordinary resistance to the lethal and mutagenic effects of most agents that damage DNA, including ultraviolet (UV) radiation and the genotoxin mitomycin C (MMC). The extremely efficient ability to repair DNA damage induced by UV and MMC has been attributed, through complementation analysis, to two putative novel repair endonucleases (UV endonuclease-α and UV endonuclease-β) that incise deinococcal chromosomal DNA at or near sites of DNA damage, thereby initiating enzymatic excision of the DNA lesions. These repair endonucleases are encoded by the *mtcA*, *mtcB* (endonuclease-α) and *uvsC*, *uvsD*, and *uvsE* (endonuclease-β) genes.

The gene encoded by *mtcA* and *mtcB* was found by DNA sequence analysis to be a single gene encoding a protein identified as the homolog of *E. coli* UvrA. UvrA is one protein of the UvrABC exonuclease complex in *E. coli* that recognizes thymine dimers and other bulky damage on nucleotide strands after UV induced DNA damage or other bulky DNA damage, such as MMC adducts. The nucleotide sequence of the *uvrA* homolog in *D. radiodurans* is over 60% homologous with *E. coli* and *M. luteus uvrA* nucleotide

sequences. The UvrA homolog in *D. radiodurans*, like *E. coli* UvrA, contains amino acid sequence for two ATP binding sites as well as two zinc binding domains and one helix-turn-helix region. Several open reading frames adjacent to and divergently transcribed from *uvrA* (*mtcA, mtcB*), were found in the location in which the single-stranded binding protein (*ssb)* is normally located. The *uvrA* operator region of *D. radiodurans* was found to be nearly identical with the *E. coli uvrA* operator region; however, the *lexA* repressor binding motif was not fully conserved. *E. coli*, *M. luteus*, *S. marcescans*, *B. abortus* and now *D. radiodurans* have homologous genes encoding *uvrA* which indicate that this gene is highly conserved among Gram positive as well as Gram negative bacteria. The presence of a *D. radiodurans* gene homologous to *uvrA* of *E. coli* suggests the existence of an enzyme complex similar to UvrABC exonuclease encoded by *uvrA*, *uvrB*, and *uvrC* genes of *E. coli*. It is unclear at the time whether *Deinococcus radiodurans* possess a *uvrB* and a *uvrC* as seen in *E. coli*.

In addition, complementation studies were performed utilizing *E. coli uvrA* in the *uvrA* mutant strain 302. The strain 302ΩpS11::*uvrA* yielded wild-type cell survival following treatments of MTC and 4-nitro-quinoline-1-oxide. The mutant strain 302 was incapable of repairing DNA damage caused by these chemicals. These studies indicate that *E. coli uvrA* can functionally replace *D. radiodurans uvrA* in repairing these forms of damage.

Finally, a 1,000 bp fragment including the 5' termini of the *uvrA* plus a short upstream segment (262 bp) was found to restore ionizing radiation resistance to the ionizing radiation mutant, IRS18. This finding suggests that the function of *D. radiodurans uvrA* may be more complex than *E. coli uvrA*, and that *D. radiodurans uvrA* may potentially play a role in ionizing radiation resistance.

IDENTIFICATION AND CHARACTERIZATION OF UVRA, A DNA REPAIR GENE OF DEINOCOCCUS RADIODURANS

by

Heidi J. Agostini

Dissertation submitted to the Faculty of the Department of
Pathology Graduate Program of the Uniformed Services
University of the Health Sciences in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy, 1996

DEDICATION

This dissertation is dedicated to my mother, Mary and my father, Jerry. Since childhood they have taught me that I can achieve any goal with dedication and hard work. From my father I inherited a persistent, stubborn, goal oriented spirit. From my mother I learned faith in God, and inherited her intuition, as well as a joyful and adventuresome spirit. Thanks Mom and Dad for always believing in me.

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These three things have I experienced and developed during my graduate studies:

Wonder:

"O Lord, our Lord, how majestic is your name in all the earth!
You have set your glory above the heavens.
From the lips of children and infants you have ordained praise
because of your enemies, to silence the foe and the avenger.
When I consider your heavens, the work of your fingers,
the moon and the stars, which you have set in place,
what is man that you are mindful of him,
the son of man that you care for him?
You made him a little lower than the heavenly beings
and crowned him with glory and honor.
You made him ruler over the works of your hands;
you put everything under his feet: all flocks and herds,
and the beasts of the field, the birds of the air,
and the fish of the sea, all that swim the paths of the seas.
O Lord, our Lord, how majestic is your name in all the earth!"

Psalm 8

Adventure:

"This hobbit was a very well-to-do hobbit, and his name was Baggins. The Bagginses have lived in the neighbourhood of the The Hill for time out of mind, and people considered them very respectable, not only because most of them were rich, but also because they never had any adventures or did anything unexpected: you could tell what a Baggins would say on any question without the bother of asking him. This is a story of how a Baggins had an adventure, and found himself doing and saying things altogether unexpected. He may have lost the neighbours' respect, but he gained - well, you will see whether he gained anything in the end."

The Hobbit

Persistence:

"Never give up. Never give up. Never give up. Never! Never! Never!"

Winston Churchill

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I. INTRODUCTION

Preface

This introduction consists of four parts. Part one contains a brief description of the general features of the Deinococcaceae family. Particular emphasis is placed on the characteristics and properties of *D. radiodurans* throughout this introduction since a thorough understanding of this species is relevant to the work presented in this dissertation. The second part is divided into two subsections. The first subsection details what specific types of damage (molecular, as well as genetic) occurs to a living bacterial cell when exposed to DNA damaging chemicals as well as different forms of radiation. This work utilized both types of treatment to inflict DNA damage on *D. radiodurans*. The second subsection describes how bacterial cells repair each of these types of insult, followed by a brief explanation of how *D. radiodurans* in particular deals with various types of DNA damage. The third part of the introduction describes the DNA repair mutants developed in *D. radiodurans* and the corresponding genes that complement the mutations identified. The last part of the introduction details the specific aims of this dissertation.

A. Overview of the *Deinococcus* Genus

There are several remarkable characteristics of the *Deinococcus* genus that are not observed in other bacteria. In this overview, we will discuss the isolation and classification of the genus, the unusual features of the cell wall and intramolecular compounds, and the key genetic characteristics of the bacteria.

1. Isolation and Classification

Deinococcus radiodurans, strain R1 the type strain of the genus Deinococcus, was first isolated in a meat canning plant in Oregon (Anderson, 1956). As part of the sterilization process, the canned meat was exposed to gamma irradiation. Some of the canned meat exhibited evidence of spoilage. A nonspore forming bacterium in the meat was

discovered which formed salmon-pink colonies on agar. This bacteria was found to require doses of 5 to 6 Mrad (50,000 to 60,000 Gy in S. I. units) of ionizing radiation for sterilization and led to the organism originally being designated *Micrococcus radiodurans* (Anderson et al., 1961), and being described in the Guinness Book of Records (1995) as the "toughest bacterium in the world". Other isolates of *D. radiodurans* were obtained from the hides of cattle that were slaughtered for the canning plant as well as from irradiated sawdust used for mushroom culture in Japan (Ito et al., 1977). A second strain of this organism was isolated as a contaminant in a hospital laboratory in Ontario, Canada. This strain was designated *D. radiodurans* SARK and has a 33% DNA homology with *D. radiodurans* R1 (Brooks et al., 1980). Several other species of Deinococcus have been isolated from various gamma irradiated materials. *D. radiopugnans* was isolated from irradiated haddock tissue (Davis et al., 1963), *D. radiophilus* from Bombay duck (Lewis, 1971) and *D. proteolyticus* from the irradiated feces of a llama (Kobatake et al., 1973), as well as from animal feed, sewage and surprisingly also from Swedish bed linens (Ito et al., 1983). It appears that Deinococcus is most frequently found in soil and soil-contaminated items (Masters, 1991). All of the above mentioned species were shown to have properties similar to those of *D. radiodurans*. Later work determined that the Deinococci were not only gamma radiation resistant but also extremely resistant to UV light (Duggan et al., 1959), with R1 being the least resistant (Fig. 1).

Originally, the above mentioned species were classified under the genus Micrococcus, but subsequent studies revealed important differences from other members of this genus, and suggested that *Micrococcus radiodurans* and related species should be reclassified into a new taxon (Baird-Parker, 1965, 1970; Thornley et al., 1965; Sleytr et al., 1973). Among these differences are unique characteristics of the cell wall structure (Thornley et al., 1965; Work and Griffiths, 1968; Sleytr et al., 1973; Lancy and Murray, 1978; Baumeister and Kubler, 1978; Thompson and Murray, 1981; Schleifer and Kandler, 1972), cell membrane components (Knivett et al., 1965; Work, 1964; Girard, 1971;

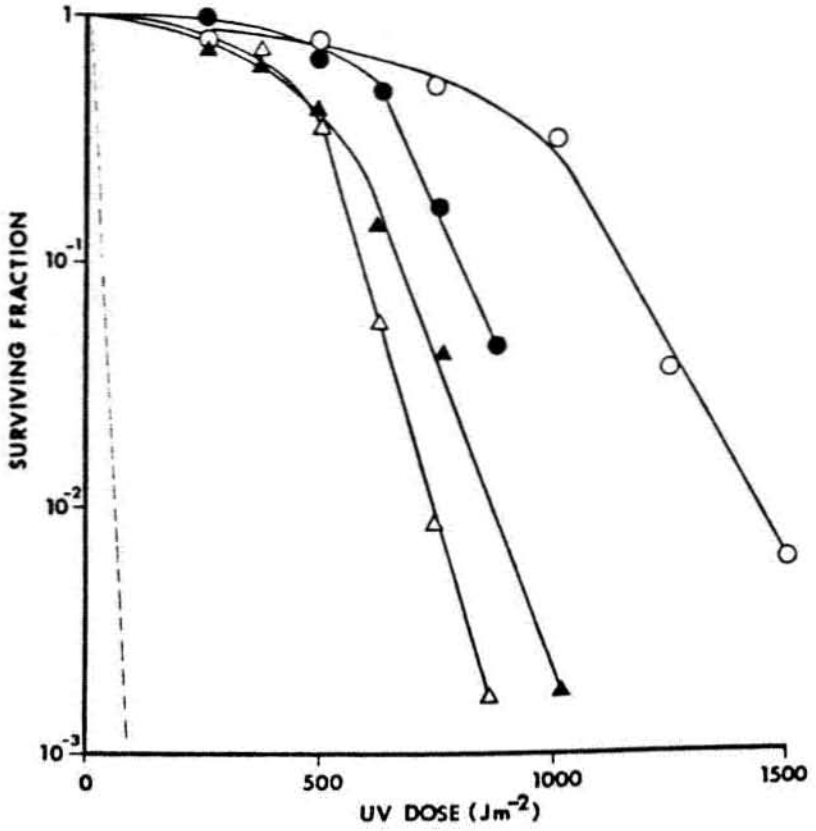


Figure 1. Ultraviolet radiation survival curves of four Deinococcus strains. ( ) *D. radiodurans,* ( ) *D. radiopugnans,* ( ) *D. radiophilus* and ( ) *D. proteolyticus* (data from Moseley, 1983). The dashed line is indicative of the UV_{254} survival of *E. coli.* (Reproduced with permission from Moseley, 1983).

Thompson et al., 1980), and degree of radiation resistance. Subsequent studies of 16s ribosomal RNA sequences confirmed that *M. radiodurans* and the other species constituted a completely different genus from Micrococcus (Brooks et al., 1980). The name Deinococcus (Gr. deinos meaning strange or unusual; Brooks and Murray, 1981), was proposed, and four species were assigned to the genus cocci: *D. radiodurans* (type species), *D. radiopugnans* (formerly *M. roseus*). *D. proteolyticus* (formerly *M. radioproteolyticus*) and *D. radiophilus* (formerly *M. radiophilus*) (Brooks et al., 1980). *D. radiodurans* R1 and *D. radiodurans* SARK (as mentioned above) are two strains of the same species *D. radiodurans*. The other genus of the Deinococcaceae is Deinobacter and the single species in this genus is *Deinobacter grandis* (Oyaiza et al., 1987; Murray, R. G. E., 1992).

The unusual structural features of *D. radiodurans* have generated some consideration of its evolutionary origins. Some investigators have suggested that Deinococcus' extraordinary DNA repair capabilities developed under selective pressure to resist elevated levels of harmful radiation and free radicals in a pre-historic environment (Berkner and Marshal, 1964). Other investigators have suggested that high levels of superoxide dismutase may have served as a defensive mechanism in the earth's early atmosphere (McCord et al., 1971; Rao and Cammack, 1981). Some investigators have even suggested that Deinococcus utilized its enhanced DNA repair capacities to survive vacuum-UV bombardment while in outer space (Hoyle and Wickramasinghe, 1981; K. Minton, personal communication).

2. The Cell Wall

D. radiodurans possesses an unusual cell envelope which has not been seen in any genus to date. Even though *D. radiodurans* stains Gram+, and is considered a Gram+ cocci, its cell wall is not typical of a Gram+ cocci. It possesses four layers exterior to the cytoplasmic membrane, each with very unusual features (Fig.2). Briefly, the plasma

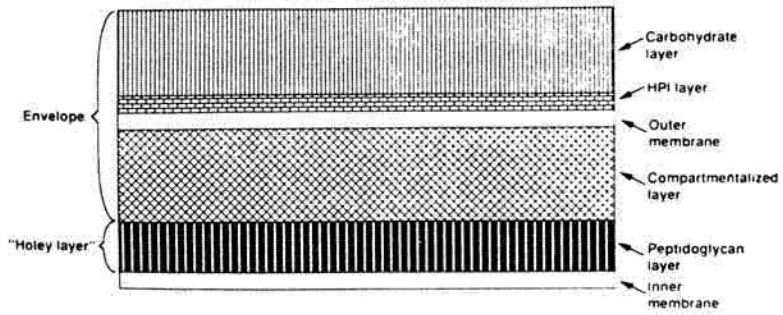


Figure 2. Schematic diagram of the cell wall of *D. radiodurans*. (Reproduced with permission from Friedberg et al., 1995).

membrane and the outer membrane are separated by a peptidoglycan layer and a compartmentalized layer. The first layer above the plasma membrane, the holey layer, is composed of a thick peptidoglycan layer and contains alanine, glutamic acid, glycine and L-ornithine instead of the more common lysine or pimelic acid (Works, 1964; Works and Griffiths, 1968). The holes or "fenestrations" in this layer are about 10 nm in diameter (Thompson and Murray, 1982). The composition of the next layer, the compartmentalized layer is unknown. The outer membrane contains some of the same lipids as the plasma membrane, but in different proportions. The next layer, the HPI (**h**exagonally **p**acked **i**ntermediated) layer of the cell consists of a protein which is arrayed in hexagonal subunits. The HPI proteins are arrayed in an interlocking hexagonal pattern and attach below to the outer membrane and laterally to other units to form the HPI layer (Baumeister and Saxton, 1982; Thompson and Boyce, 1981). The gene for the major HPI protein has been cloned, sequenced and characterized (Peters and Baumeister, 1986, 1987).

The four layers of the cell wall can be broken into two main sections: the "holey layer" and the envelope (Fig 2). The envelope, or sheath of the deinococci is unusual and completely unlike other Gram+ bacteria such as *S. aureus* or *B. licheniformis* which have a single homogeneous, thick component external to the plasma membrane. The sheath is also different from those of most Gram- bacteria such as *E. coli*, which consist of a peptidoglycan and an outer membrane layer exterior to the plasma membrane. The sheath does appear to share some similarities to those of blue green algae (Ris et al., 1981). One striking feature is that the sheath surrounds groups of cells (usually two) and forms on the surface of daughter cells as they separate (Thornley et al., 1965; Work et al., 1968; Sleytr et al., 1973). *D. radiodurans*, but no other species of the genus, possesses an HPI layer, arranged in a hexagonal array (Brooks and Fox, 1980; Baumeister and Kubler, 1978; Baumeister and Zingsheim, 1981; Lancy and Murray, 1977; Thompson and Murray, 1981, 1982; Sleytr et al., 1973). On some strains, including *D. radiodurans*, the outermost layer is a thick, dense carbohydrate layer (Baumeister et al., 1981).

The lipids of the deinococci are complex in composition and distinct from those found in most other bacteria (Lewis et al., 1974; Thompson and Murray, 1980). *D. radiodurans* does not possess the usual hydroxy fatty acids that comprise part of the lipopolysaccharide of the outer membrane of Gram- bacteria. Nor does *D. radiodurans* possess the conventional phospholipids found in the plasma and outer membranes of the bacteria, but instead the membranes are constructed of a novel phosphoglycolipid [2'-O-(1,2,-diacyl-sn-glycero-3-phospho)-3'-O-(galactosyl)-N-D-glyceroyl alkyamine] and several other unidentified glycolipids, glycophospholipids and phospholipids (Brooks et al., 1980; Hansen, 1978).

3. Intracellular Compounds

Unusually high levels of certain intracellular molecules are observed in *D. radiodurans*. However, satisfactory explanations for these unusually high levels have not been found, and it remains unclear whether these compounds have any effect on radioresistance.

Initial research suggested that the carotenoid (red) pigment might have an effect on radioresistance. In fact, some pigmentless strains were isolated which were sensitive to UV and gamma radiation (Moseley, 1963; Okazawa and Matsuyama, 1967). However this finding was not consistent with other pigmentless strains, which were just as resistant as the wild-type (Moseley, 1963; Okazawa and Matsuyama, 1967; Lewis et al., 1974).

The high sulphydryl content of the bacterium was also implicated in radioresistance, and led to the isolation of a "factor" that was claimed to be responsible for radioresistance (Bruce, 1964). This "factor" protected *E. coli* from X-irradiation and was postulated to play a role in DNA repair (Goldstein et al., 1978). Others could not corroborate these findings (Serianni and Bruce, 1968).

The unusually high concentration of manganese around the chromosomes of *D. radiodurans* was thought to play a role in radioprotection due to its ability to reduce the

yield of UV photoproducts, specifically thymine dimers (Leibowitz et al., 1976). It has reported that manganese decreases radiation resistance and induces cell division and superoxide dismutase levels in *D. radiodurans* (Chou and Tan, 1990). Superoxide dismutase is a free radical scavenger, which reduces free radicals around the chromosomes and thereby helps to prevent DNA damage. In addition, the UV endonuclease-β, which is active in thymine dimer repair in *D. radiodurans* (Evans and Moseley, 1983), has an unusually high requirement for manganese (Kitayama and Matsuyama, 1977; Chou and Tan, 1990). However, a typical level of about 1% of thymine in DNA is present as thymine-thymine dimers at 500 J/m^2 as in other bacteria, which is a sublethal dose for *D. radiodurans* but not other bacteria (Boling and Setlow, 1966).

4. Genetic Features

a. DNA Content. Species of the Deinococcaceae have a high GC content ranging from 65-71% (Brooks et al., 1980; Moseley et al., 1964). *D. radiodurans* apparently lacks methylated bases such as 5-methylcytosine or 6-methyladenine (Schein, 72; Storl et al., 1979), although some form of modification must protect their DNA from endogenous restriction enzymes such as Dra I and Mra I (Table I) (Mackay et al., 1985; Al-Bakri et al., 1985; Purvis et al., 1983; Wani et al., 1982). The other species of *Deinococcus* also possess resident restriction enzymes (as listed in Table 1).

b. Chromosomes. The deinococcal chromosome is about 2,000 kb and contains multiple identical copies of its chromosome, ranging from four in a resting cell to as many as ten in an exponentially growing one (Tigari and Moseley, 1980; Driedger et al., 1970; Hansen, 1978; Harsojo et al., 1971; Moseley and Evans, 1981). It has been suggested that Deinococcus' multiple chromosomes may play a critical role in its radiation resistance (Krasin and Hutchinson, 1977; Minton, 1994; Minton and Daly, 1995). However, the number of chromosomes alone does not seem to increase radiation resistance, as demonstrated in the case of *Azotobacter vinelandi*, which has more than 40 chromosomes

Table 1. Cellular features of several Deinococcus strains.

Isolate	*Deinococcus radiodurans*	*Deinococcus radiopugnans*	*Deinococcus radiophilus*	*Deinococcus proteolyticus*	*Deinobacter grandis*
Morphology	Cocci	Cocci	Cocci	Cocci	Cocci
Gram reaction	+	+	+	+	-
G + C content	68	66-68	65	67	69
B-gal assay	-	+	-	-	ND
Transformability	+	-	-	-	ND
Restriction endonucleases	*Mra*I	*BstE*II* *Pvu*I* *Xho*I*	*Dra*I *Dra*II *Dra*III	ND	ND
Fenestrated peptidoglycan	+	+	-	-	-
Natural plasmids	pS16 (R1) pUE10 (Sark) pUE11 (Sark)	pUE30 pUE31	pUE1 pUE2 pUE3	pUE20 pUE21	pUE15

* Restriction enzymes from isolates of *D. radiopugnans* that are isoschizomers to the enzymes shown here.
ND. Not determined. (Reproduced with permission from Moseley, 1983).

and is very radiosensitive (Sadoff et al., 1979). Furthermore, the extent of radiation resistance in *D. radiodurans* is not significantly changed when chromosome multiplicity is varied between four to ten (Hansen, 1978; Harsojo et al., 1971).

The arrangement of *Deinococcus*' multiple chromosomes may be important in explaining its radiation resistance (Minton, 1994; Minton and Daly, 1995). Earlier studies have suggested that the deinococcal chromosome is attached to the plasma membrane, as in *E. coli*, and that these attachments may be important in the process of repair of radiation damage (Burrell et al., 1971; Dardalhon-Samsonoff et al., 1975, 1980; Driedger and Grayston, 1970). However, these studies were inconclusive and difficult to duplicate. A recent proposal is that the multiple chromosomes are linked together by either Holliday junctions (Minton, 1994) or proteins (Moseley, 1966), which may facilitate homologous recombination between chromosomes. Further research in this area will help clarify the roles of multiple chromosomes in the repair of DNA damage.

c. Plasmids. Plasmids have been found in all four strains of the *Deinococcus* genus (Mackay et al., 1985; Smith et al., 1988). These plasmids are usually low in copy number and larger than 20 kb (Mackay et al., 1985). The *D. radiophilus* plasmid pUE1 and two *D. radiodurans* Sark plasmids (pUE10 and pUE11) have been cloned in *E. coli* (Smith et al., 1990; Mackay et al., 1985; Smith et al., 1989). The pS16 plasmid of *D. radiodurans* strain R1 is isolated in low amounts and may be an episome (Smith et al., 1988, 1990). The plasmid pS16 is homologous to the *D. radiodurans* chromosome and to the cryptic plasmid pUE11 found in strain Sark (Smith et al., 1990).

d. Transformation. *D. radiodurans* R1 and Sark are naturally transformable, meaning that they not only take up plasmid DNA but that they readily recombine homologous DNA into the genome. The R1 strain is equally competent throughout logarithmic growth, but stationary phase cells are much less competent (Tigari and Moseley, 1980). Competence throughout logarithmic growth occurs in *Neisseria meningitidis* , but is not observed in *B. subtilis, Haemophilus influenzae* , or *Streptococcus*

pneumoniae (Stewart and Carlson, 1986). Transformation of *D. radiodurans* is linearly dependent on DNA concentration up to 1 ug/ml (Moseley et al., 1991). Although single- and double-stranded DNA are taken up with equal efficiency by R1, double-stranded DNA is 10-fold more active in transformation. Uptake is not limited to deinococcal DNA, because foreign DNA effectively competes for uptake with deinococcal DNA (Tigari et al., 1980).

e. Repeated sequences. Recently, Lennon et al. (1991) discovered the presence of repeated sequences in *D. radiodurans* R1 and SARK. These sequences ranged from 150 to 192 bp, and the difference in size depended on the presence of two 21 bp regions within the sequence. Within the 192 bp sequence, there are four areas of dyad symmetry and a direct repeat. The whole sequence is highly conserved, and at least 40 copies are present in each chromosome. The possible function of the sequence has not been determined, but the authors suggested that these repeated sequences may be involved in the regulation of DNA damage-inducible genes. Within the reiterated sequence, an open reading frame (ORF) with an upstream Shine-Dalgarno sequence is also present; this ORF may represent a leader sequence for transport (Lennon et al., 1991).

f. Shuttle plasmids between *D. radiodurans* and *E. coli.* Shuttle vectors, which replicate and express drug resistance in both *D. radiodurans* and *E. coli,* have been constructed from plasmids pUE10 and pUE11 of *D. radiodurans* strain SARK (Smith, M.D., 1988,1990,1991). Recently, two other shuttle vectors, pMD66 and pMD68, have been constructed to study recombination between plasmids and chromosomes (Daly et al., 1994).

5. Summary

From the description of these unusual structural features of *D. radiodurans* , one would suspect that many structural components may contribute to *Deinococcus*' extreme radioresistance, by reducing the degree of damage inflicted. However, many investigators

have shown that *D. radiodurans* cannot prevent DNA damage to any greater degree than *E. coli*, suggesting that these structural features play minor roles, if any, in *D. radiodurans'* radioresistance (Moseley, 1983). Since *D. radiodurans* is capable of repairing DNA damage far more efficiently than *E. coli* or other bacteria (Moseley, 1983), *Deinococcus'* repair pathways can be expected to play a far more central role in its extreme radioresistance. Thus, the following material discusses sources of DNA damage, bacterial DNA repair pathways, and the unique repair capabilities of *D. radiodurans*.

B. DNA Damaging Agents

Before we can proceed with a description of the DNA repair pathways in *D. radiodurans* we must first understand the process of molecular damage in cells as a result of radiation and chemical exposure.

1. Visible light and low-frequency UV radiation

Most of the solar radiation that reaches the earth's surface is in the form of visible light (400-700 nm), which is absorbed by chromophores (van Liere et al., 1982). An excited chromophore either transfers the energy to a target molecule or forms radicals such as superoxide, which then can degrade other molecules in the cell. As stated earlier, *D. radiodurans* possesses large quantities of carotenoid pigments and superoxide dismutase; these compounds help protect it from the harmful effects of visible light (Carbonneau et al., 1989; Lewis et al., 1974; McCord et al., 1977; Moseley, 1963).

Low-frequency UV radiation (290-400 nm), like visible light, is first absorbed by a chromophore, which then causes damage by transferring energy to the target or by creating free radicals (Eisenstark, 1989). Since low-frequency UV radiation is more energetic than visible light, the damage per photon absorbed is correspondingly higher. *D. radiodurans* is unusually sensitive to low-frequency UV radiation, even if given a sublethal dose before a challenge dose (Caimi et al., 1986) which would usually result in a protective priming effect.

2. High-frequency UV radiation

High-frequency UV radiation (200-290nm) is readily absorbed by DNA (260 nm corresponds to the absorption peak of DNA) and RNA, and damages both species by dimerizing bases and creating other photoproducts that interfere with replication of the DNA and its transcription. High-frequency UV radiation comprises a small but significant fraction of solar radiation at the surface of the earth. High-frequency UV radiation is also generated by germicidal lamps, which are commonly used to sterilize surfaces.

a. Pyrimidine dimers. At the molecular level, when DNA is exposed to wavelengths approaching 260 nm, adjacent pyrimidines become covalently linked in what is referred to as a cyclobutane dipyrimidine or pyrimidine dimer (Fig. 3). These cyclobutane dimers are considered bulky, helix-distorting lesions which are noncoding and whose presence results in a block in replication (Chan et al., 1985; Hayes et al., 1971; Witkin, 1976). Pyrimidine dimers are not limited to thymines, and can form between cytosines as well.

The relative incidence of the various pyrimidine dimers (T-T, C-T, C-C) is primarily influenced by the nucleotide composition of an organism. The table below lists the distribution of dimers in bacteria with various concentrations of nucleotides (Table 2). *D. radiodurans* possesses a high G+C content, and its dimer distribution is similar to *M. luteus*. For many years, dimer formation was thought to occur nonspecifically and randomly throughout the genome. However, recent experiments have contradicted this hypothesis, and suggest that the yield of dimers in DNA is influenced by sequence context. In one experiment, the frequency of dimer formation in the sequence TGAAA**TT**G**TT**AT was significantly higher than that observed in either of the sequences **CT**ATA**TT**GA**TT**A and TGGAA**TT**GTGAG (Gordon et al., 1982). Furthermore, recent work has revealed "hot spots" for DNA damage, where many bases are damaged in a localized manner at or within genes on the transcribed strand (Friedberg et al., 1995).

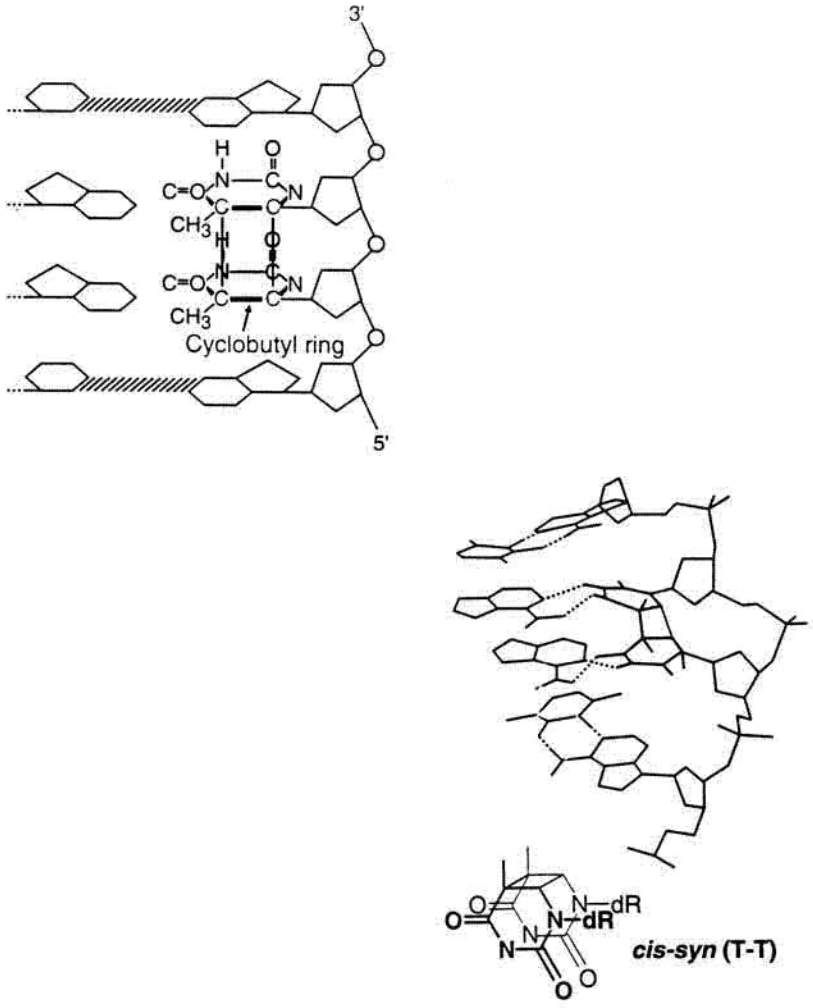


Figure 3. The cyclobutane pyrimidine dimer and its proposed three dimensional orientation. (Reproduced with permission from Freidberg et al., 1995)

Table 2. Distribution of pyrimidine dimers in UV irradiated DNA.
(Reproduced with permission from Freidberg et al., 1995)

Source of DNA	Wavelength (nm)	Dose (J/m^2)	Distribution of dimers (%)		
			C-C	C-T	T-T
Haemophilus influenzae (high A+T)	265	2×10^2	5	24	71
	280	4×10^3	3	19	78
E. coli (G+C ≈ A+T)	265	2×10^2	7	34	59
	280	4×10^3	6	26	68
M. luteus (high G+C)	265	2×10^2	26	55	19
	280	4×10^3	23	50	27

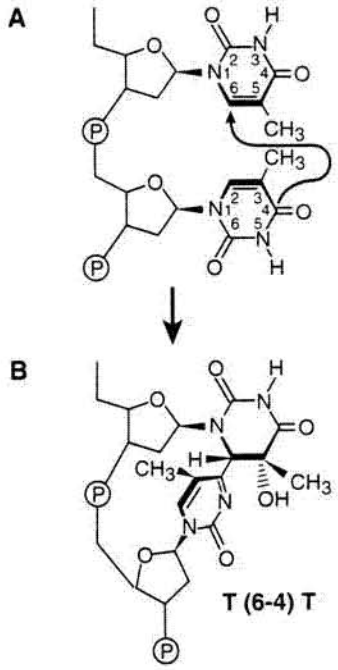


Figure 4. The formation of a thymine-thymine (6-4) photoproduct.
(Reproduced with permission from Freidberg et al., 1995)

b. The (6-4)-photoproduct. The pyrimidine-pyrimidone (6-4) photoproduct, often called the (6-4) photoproduct, is another type of dimer formed between bases on the same strand. In general, this type of damage occurs less frequently compared with thymine dimers, and is usually seen in decreasing frequency from TC-CC-TT (Fig. 4). In *D. radiodurans* , however, (6-4) photoproducts comprise about 17% of overall DNA damage, approaching the percentage of thymine dimers formed (Moseley, 1983).

c. Thymine glycols and cross-links. The formation of thymine glycol or 5,6-dihydroxydihydrothymine lesions also occurs frequently in response to UV irradiation. Such lesions comprise an important type of ionizing radiation-induced DNA base damage, but can also result from UV radiation (Demple et al., 1982; Yamane et al., 1967). *E. coli* contains an enzyme, thymine glycol DNA glycosylase, that catalyzes the excision of this type of base damage from DNA (Demple et al., 1980). An equivalent enzyme in *D. radiodurans* has been detected (Munn et al., 1994). UV radiation can also result in the cross-linking of DNA to proteins (Peak and Peak, 1986), and infrequently causes interstrand cross-linking of DNA to DNA (Marmur and Grossman, 1961). In summary, UV radiation predominantly damages DNA through the formation of thymine dimers, but also in the formation of (6-4) photoproducts, thymine glycols, and DNA-protein cross-links as well as DNA interstrand crosslinks. All of these types of damage are removed most efficiently by the nucleotide excision repair (NER) pathway, and are discussed further below.

3. Ionizing Radiation Damage

Ionizing radiation of solar origin is not sufficiently intense to cause significant DNA damage. Instead, the highest concentrations of ionizing radiation encountered by bacteria are from various radioactive deposits and man-made sources, usually a radioactive ^{60}Co source (Moseley, 1983). A gamma ray interacts with a cell by transferring energy to

electrons and raising them to an excited state. If the energy is sufficiently great, the negatively charged electron can escape from the molecule, leaving a positively charged ion. The ejected electron moves through surrounding material and dissipates its energy by creating further excited molecules and positive ions. Eventually the electron is captured by a positive ion or is trapped by a structure to form a negative ion. Ionizing radiation is characterized by this ability to create positive and negative ions.

Ionizing radiation acts directly on the cell through chemical events resulting from increased energy in target molecules, such as the ejection of electrons from atoms in the DNA. The indirect effects of ionizing radiation usually result from hydroxyl radicals, generated by the radiolysis of water reacting with DNA in the cell. Ionizing radiation tends to damage all molecules in the cell to a roughly equal extent, in contrast to most other forms of chemical or physical agents.

Under aerobic conditions, reaction with O_2 can result in various ring-saturated derivatives, particularly thymine glycols (5,6-dihydroxy-5,6-dihydrothymine) (Fig. 5). The saturated ring can undergo further degradation and give rise to products such as methyltartronylurea, 5-hydroxyhydantoin, N-formamidourea, and urea (Fig. 5), all of which have deleterious effects on the cell.

In contrast to other agents that generate reactive oxygen species, ionizing radiation also results in the formation of DNA damage clusters (Hutchinson, 1985; Ward, 1988). In this case, a single energy deposition event causes several radical reactions in the immediate vicinity, and results in multiple damaged bases and molecules in close proximity. The clustered destruction of DNA caused by ionizing radiation is fundamentally different from the "hot spots" associated with high-frequency UV radiation. In UV-induced DNA damage, the "hot spot" cluster of damage results from several photons depositing energy on the DNA and causing the formation of thymine dimers and other photoproducts.

Most of the lethal effects of ionizing radiation can be attributed to strand breakage, and in particular to double-strand breaks (Hutchinson, 1985; Iliakis, 1991; von Sonntag;

Figure 5a. Formation of thymine glycol by ·OH radical attack at thymine.(Figs. 5a and 5b reproduced with permission from Freidberg et al., 1995).

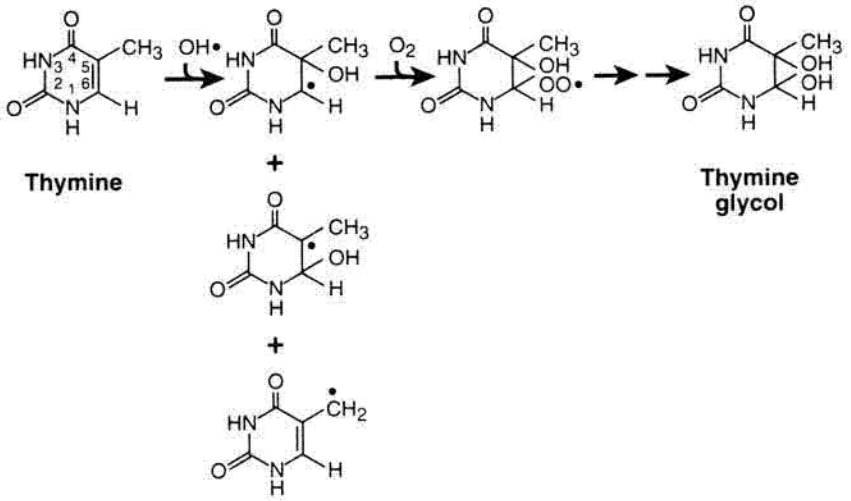
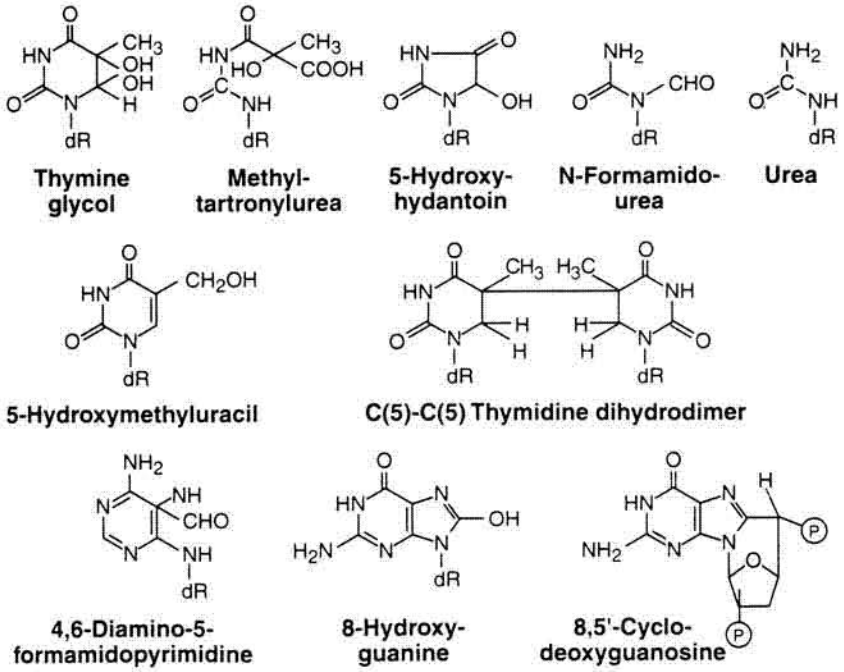


Figure 5b. Examples of base damage induced by ionizing radiation.



1987). Single-strand breaks are initiated by radical formation of deoxyribose following the loss of a hydrogen atom. These lesions are not easily repaired, because the strand breaks result in damaged 3' termini in which the OH groups are missing. Although the 5' ends still possess phosphate groups (Bopp and Hagen, 1970; Henner et al., 1982), DNA ligase is unable to operate (Obe et al., 1992; von Sonntag, 1987) and therefore requires more complicated means of repair. In summary, random energy deposition by ionizing radiation causes a wide array of different DNA lesions.

4. Chemical Damage

Although a multitude of DNA altering chemical agents have been discovered, it is useful to focus primarily on those agents which have been used in this research.

a. Alkylating Agents. Alkylating agents are electrophilic compounds with affinity for nucleophilic centers in organic molecules (Lawley, 1966; Loveless, A. 1966; Roberts, 1978; Ross, 1962). Monofunctional alkylating agents have a single reactive group and interact with a single nucleophilic center in DNA. Bifunctional alkylating agents possess two reactive groups that can react with two separate DNA sites. The sites most commonly attacked by monofunctional agents are the following (Fig. 6; Roberts, 1978; Singer, 1986; Singer et al. 1983; Singer et al., 1982). In general, the ring nitrogens are more nucleophilic than the ring oxygens, with the N^7 position of guanine and the N^3 position of adenine being the most frequently attacked (Fig. 6) (Roberts, 1978). Mitomycin-C, a chemical used in this work, is a mono- and bifunctional alkylating agent which is capable of binding to all the N and O sites of a deoxyribonucleotide and causes major helical distortions as well as multiple cross-links between DNA strands. This type of damage results in a complete block in replication and transcription (Lawley, 1966; Loveless, 1966).

b. Cross-linking agents. Interstrand cross-links comprise an important class of chemical damage to DNA, since such cross-links prevent DNA strand separation and can

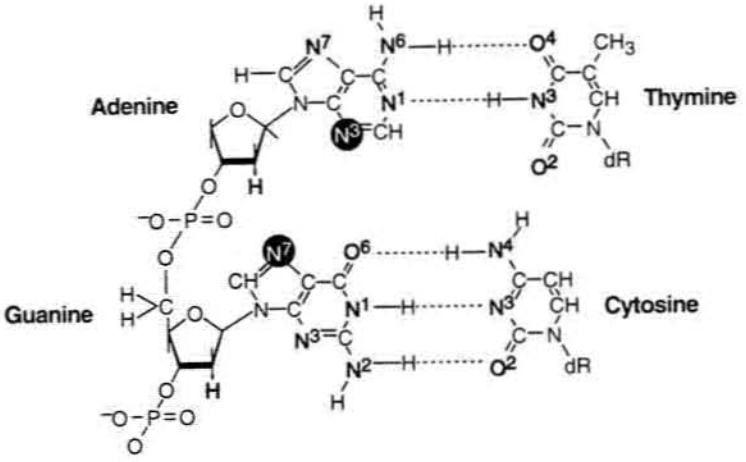


Figure 6. Nucleotide centers in DNA that are most reactive to alkylating agents. (Reproduced with permission from Freidberg et al., 1995)

result in a complete block to DNA replication and transcription. The DNA cross-linking agents used in this work are mitomycin-C, and photoactivated psoralens. The effects of other compounds such as nitrous acid (Geiduschek, 1961), nitrogen mustard and sulfur mustard (Chun et al., 1969; Fox et al., 1980; Kohn et al., 1966) and various platinum derivatives [such as cis-platinum (II) diaminodichloride] (Chu, 1994; Eastman, 1987) have been studied in *D. radiodurans* (Moseley, 1983). In addition, UV radiation at about 254 nm (Love et al., 1986; Marmur et al., 1961) and ionizing radiation (Lett et al., 1961; von Sonntag, 1987) can also result in the formation of DNA cross-links as minor products of DNA damage.

c. Psoralens. The psoralen-plus-UV light reaction is highly specific for native DNA, and forms stable cross-links between DNA strands. Psoralen monoadducts to pyrimidine bases are formed about three times as frequently as cross-link damage (Cole, 1971; Pathak et al., 1974; Sinden et al., 1978). However, cross-link damage appears to be primarily responsible for cell death in bacteria, bacteriophages, and eukaryotic cells with photoactivated psoralen (Ashwood-Smith et al., 1977; Averbeck, 1989; Brendel et al., 1984; Cole, 1971)

d. 4-Nitroquinoline 1-Oxide. DNA damage induced by 4-nitroquinoline 1-oxide (4NQO) is often referred to as "UV radiation-like" because this chemical produces bulky base damage of the type that, like cyclobutane pyrimidine dimers and (6-4) photoproducts, distorts the DNA backbone and is repaired principally by the nucleotide excision repair system (NER). 4NQO is first converted to the carcinogen 4-hydroxyaminoquinoline 1-oxide. This intermediate is apparently further activated by various compounds, ultimately resulting in a N^2 adduct which appears to be the major lesion that accounts for 50 to 80% of all quinoline base adducts (Menichini et al., 1989). 4NQO treatment can also result in the formation of 8-hydroxyguanine (Kohda et al., 1986) and leads to a significant amount of strand breakage (Galiegue-Zouitina et al., 1985).

C. DNA Repair Mechanisms

1. Direct Reversion of DNA damage

One of the simplest and easiest ways to repair DNA damage is when a single enzyme composed of a single polypeptide chain catalyzes a single-step reaction which restores the genome to its normal state. One would imagine that there are many biochemical and enzymatic advantages to having such a system as opposed to having a multistep pathway to repair a single type of damage. In addition, the potential for introducing errors into the nucleotide sequence would be reduced or eliminated. In the next section four examples of direct reversal of DNA damage, all of which are catalyzed by single polypeptides, will be examined (Table 3).

a. Photoreactivation of DNA. Since cyclobutane pyrimidine dimers constitute major sources of base damage to cells it is understandable that cells have evolved multiple processes for removing this damage. Photoreactivation (PR) is a light-dependent process which involves the enzyme catalyzed monomerization of pyrimidine dimers. The photoreversible process proceeds as follows: After a dimer forms, following UV irradiation, a photolyase (49 kDa) protein binds to the dimer. In the presence of light, the photolyase (via catalytic activity of two chromophores) monomerizes the dimers and is released from the DNA (Fig. 7).

DNA photolyase was first identified and characterized in *E. coli* in 1958 (Rupert et al., 1958). There are very low amounts of this protein per cell (about 10-20 molecules) (Harm et al., 1968). Interestingly, photoreactivation repair of pyrimidine dimers has not been demonstrated in *D. radiodurans* (Moseley and Laser, 1965). For example, in one study, increased survival of two UV-irradiated UV-sensitive mutants *D. radiodurans*, rec30 and strain UV47, did not occur following photoreactivation treatment (Moseley and Copland, 1975).

Table 3. Three types of cellular responses to DNA damage.
(Reproduced with permission from Freidberg et al., 1995)

Response	Mechanism
Reversal of DNA damage	Enzymatic photoreactivation Repair of spore photoproduct Repair of O^6-alkylguanine, O^4-alkylthymine, and alklyphosphotriesters Ligation of DNA strand breaks
Excision of DNA damage	Base excision repair Nucleotide excision repair Mismatch repair
Tolerance of DNA damage	Replicative bypass of template damage with gap formation and recombination Translesion DNA synthesis

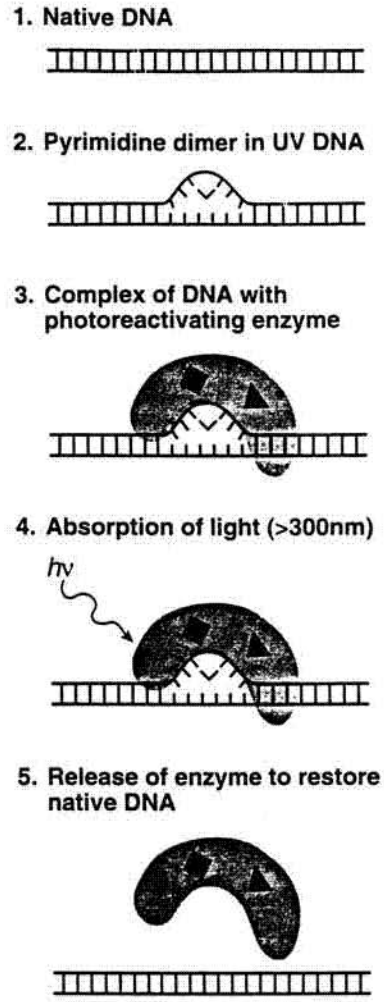


Figure 7. Schematic illustration of the enzyme-catalyzed monomerization of pyrimidine dimers by DNA photolyase. (Reproduced with permission from Freidberg et al., 1995)

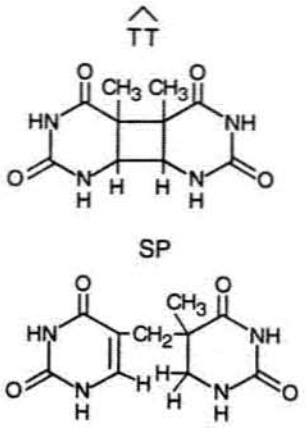


Figure 8. Structure of the spore photoproduct. (Reproduced with permission from Freidberg et al., 1995)

b. Repair of Spore Photoproduct. In *Bacillus subtilis*, spores usually form due to the depletion of one or more nutrients. Spores have no detectable metabolism and can usually survive for extended periods of time. During the time that they are exposed to the environment they may sustain damage to their genome which is usually repaired when they germinate. The repair process which operates in germination is specific for the repair of spore-specific UV-induced DNA damage termed spore photoproduct (SP) (Fig. 8; Setlow, 1992). *D. radiodurans* does not form spores and therefore does not possess this repair process (Moseley, 1963).

2. Repair by methods involving single proteins.

a. Repair of Alkylation Damage. In this instance chemical agents cause damage to DNA bases and to the phosphodiester linkages in the sugar-phosphate backbone. The most common product of DNA damage is the addition of a methyl group to the adenines and guanines. Certain mutagenic monofunctional alkylating agents, such as methyl-N-nitro-N-nitrosoguanidine (MNNG), N-methyl-N-nitrosourea (MNU), and to a lesser extent methyl methanesulfonate (MMS), react with DNA to produce both O-alkylated and N-alkylated products. O^6-alkylguanine and O^4-alkylthymine are potentially mutagenic lesions because they can mispair during semi-conservative DNA synthesis (Coulondre et al., 1977; Loveless, 1969, Singer et al., 1990). The *E. coli* enzyme which removes methyl groups from the O^6 position of guanine was originally designated as O^6-methylguanine-DNA methyltransferase. It is also sometimes called Ada protein (Samson, 1992).

Repair of alkylation damage in *D. radiodurans* is observed. In fact the wild-type strain is able to repair potentially lethal damage caused by the bifunctional alkylating agent mitomycin C (MTC), the monofunctional alkylating agents decarbamoylmitomycin C (DCMTC), MMS, ethyl methane sulfonate (EMS), and MNNG (Moseley and Copland, 1976). It is unclear whether the wild-type strain excises these modified bases (O^6-

methylguanine, 7-methylguanine and 3-methyladenine) from its DNA by a glycosylase alone or with an apurinican endonuclease (Tempest, 1978).

Some of above mentioned chemicals cause multiple types of DNA damage which complicate the understanding of the repair processes involved. For instance, MTC causes monoalkylation damage as well as interstrand cross-links. The alkylation damage can theoretically by recognized by the NER pathway as well as a glycosylase or an apurinic endonuclease. The cross-links formed are always repaired by the NER pathway and recombination working in concert with each other. Thus, one can understand the problems one may encounter when characterizing a mutant that involves several overlapping pathways. The *D. radiodurans* mutant strain, 302 (*mtcA*) (the subject of this work) is 40 times as sensitive as the wild-type strain to the lethal effects of MTC and DCMTC, and the mutagenic effects of MMS, EMS and MNNG (Moseley and Copeland, 1978). It has been determined that the lethal effects of MTC and DCMTC are due to strain 302's inability to recognize or excise cross-link damage (Tempest, 1978). The mutability of strain 302 to the methylating agents MMS and MNNG is probably caused by the strain's inability to excise the mispairing base O^6-methylguanine (Tempest, 1978). For a more detailed explanation see Results section.

b. Repair of single-strand breaks by direct rejoining. Ionizing radiation, primarily X-rays and gamma rays cause the hydrolysis of phosphodiester bonds in duplex DNA. In most cases, the repair of DNA strand breaks requires various processes that remove this damage before the ends can be rejoined. In *E. coli*, and possibly in other prokaryotes including *D. radiodurans* some single-strand breaks in DNA produced by ionizing radiation are repaired by simple rejoining of the ends via a DNA ligase (Jacobs et al., 1972) (Fig. 9). DNA ligase is a highly specific enzyme which is ubiquitous in its distribution and plays a role in most known biochemical pathways that require the rejoining of strand breaks in DNA (Kornberg et al., 1992; Lehman, 1974). The enzyme from *E. coli* has an absolute requirement for NAD and for Mg^{2+} as cofactors (Kornberg et al., 1992;

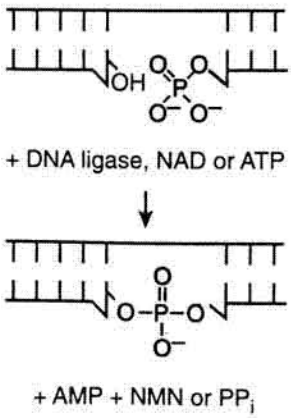


Figure 9. Repair of single strand breaks by DNA ligase. (Reproduced with permission from Freidberg et al., 1995)

Lehman, 1974). All DNA ligases require free ends in duplex DNA with no missing nucleotides at the site of the break and the presence of adjacent 3' OH and 5' P termini (Kornberg et al., 1992). Thus, only strand breaks with these particular characteristics produced by DNA damaging agents such as ionizing radiation can be repaired by direct reversal.

For example, in an *in vitro* reaction, the incubation of irradiated DNA with the enzyme DNA ligase resulted in the loss of a fraction of the total DNA strand breaks as measured by sedimentation velocity in alkaline sucrose gradients (Jacobs et al., 1972). It is very possible that a similar observation seen in *D. radiodurans* (Daly, 1994) may be due to an as yet unidentified DNA ligase. In this case, after exposure of *D. radiodurans* to a high dose of ionizing radiation, a small upward shift in the molecular weight of the resultant DNA fragments as observed on an agarose gel suggests that a ligation process of some ends of the DNA fragments may be occurring.

In each of the four examples discussed above, the reversal mode of DNA repair is kinetically advantageous since it presumably occurs more rapidly than multistep biochemical pathways such as excision repair. In general, these processes would tend to be relatively error free because of their high degree of specificity.

3. Base Excision Repair

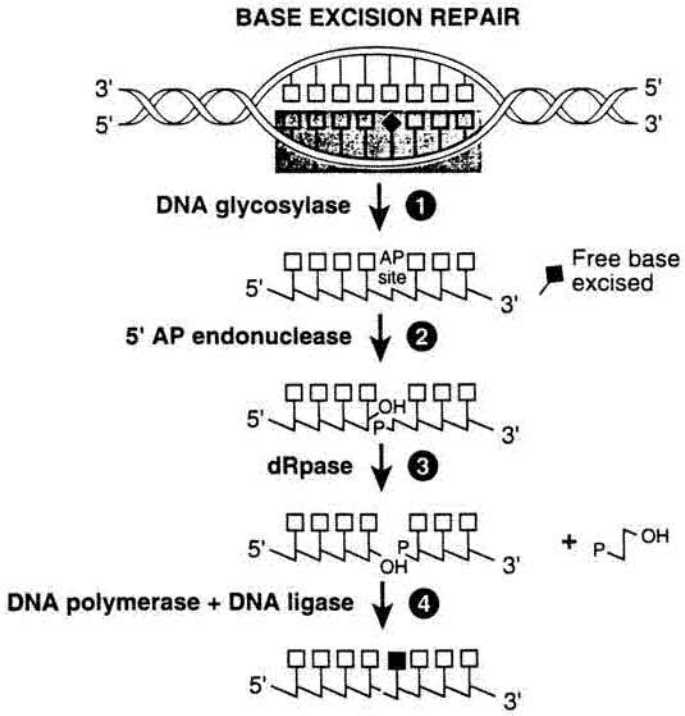
Genomes which contain individual damaged or extraneous bases (e.g., uracil) are removed by a process that utilizes two enzyme activities, glycosylase and apurinic-endonuclease. In genomes with mispaired bases, the mispaired base is excised via a mismatch repair-specific endonuclease.

a. Glycosylases, AP endonucleases. When certain types of DNA damage are encountered (Fig. 10), DNA glycosylases are utilized by the prokaryotic and eukaryotic cell, to excise the damaged base. Glycosylases act by cleaving the glycosylic bond

Table 4. DNA glycosylases. (Reproduced with permission from Freidberg et al., 1995)

Enzyme	Substrate	Products
Ura-DNA glycosylase	DNA containing uracil	Uracil + AP sites
Hmu-DNA glycosylase	DNA containing hydroxymethyluracil	Hydroxymethyluracil + AP sites
5-mC-DNA glycosylase	DNA containing 5-methylcytosine	5-methylcytosine + AP sites
Hx-DNA glycosylase	DNA containing hypoxanthine	Hypoxanthine + AP sites
Thymine mismatch-DNA glycosylase	DNA containing G·T mispairs	Thymine + AP sites
MutY-DNA glycosylase	DNA containing G·A mispairs	Adenine + AP sites
3-mA-DNA glycosylase I	DNA containing 3-methyladenine	3-Methyladenine + AP sites
3-mA-DNA glycosylase II	DNA containing 3-methyladenine,7-methylguanine, or 3-methylguanine	3-Methyladenine, 7-methylguanine, or 3-methylguanine + AP sites
FaPy-DNA glycosylase	DNA containing formamidopyrimidine moieties, or 8-hydroxyguanine	2,6-Diamino-4-hydroxy-5-*N*-methylformamido-pyrimidine and 8-hydroxyguanine + AP sites
5,6-HT-DNA glycosylase (endonuclease III)	DNA containing 5,6-hydrated thymine moieties	5,6-Dihydroxydi-hydrothymine or 5,6-dihydrothymine + AP sites
PD-DNA glycosylase	DNA containing pyrimidine dimers	Pyrimidine dimers in DNA with hydrolyzed 5' glycosyl bonds + AP sites

Figure 10. Schematic representation of base excision repair. (Reproduced with permission from Freidberg et al., 1995)



between the damaged base and the undamaged ribose. Table 4 lists the various types of glycosylases that are utilized by *E. coli*. When the damaged base is excised, an apyrimidinic or apurinic site forms that must be further processed by an AP-endonuclease (Fig. 10). The resulting gap is degraded by a DNA deoxyribophosphodiesterase, producing a single nucleotide gap that is subsequently repaired by DNA polymerase and DNA ligase (Freidberg et al., 1995). Although an AP-endonuclease has been purified in *D. radiodurans* (Masters, 1991), the sequence encoding this gene product has not been identified. *D. radiodurans* AP-endonuclease appears to possess exactly the same functions as the *E. coli* homolog, except that it doesn't require Mn^{2+}.

b. Mismatch Repair. Mismatched bases can arise by several processes, the most important of which is replication error. In this case, the correct base of the mispair is located on the parental strand of the newly replicated DNA, and correction of the mispair is essential for fidelity of genetic information. Mismatched DNA also occurs when a heteroduplex is formed between two homologous chromosomes during recombination. One or both of these homologous DNAs may contain mutations as a result of evolutionary divergence. Repair of mismatched bases is a complex process, but thorough investigation has isolated and characterized several genes and their corresponding proteins that are involved in the resolution of mismatched bases. The genes mutH, L, S, and uvrD apparently are required for mismatch repair (Cole, 1973; Shiota et al., 1988) in *E. coli*. However, no mismatch repair genes have been identified in *D. radiodurans*.

4. Multi-Protein Nucleotide Excision Repair

When genomes contain many different types of base damage not subject to glycosylases, a multiprotein nucleotide excision repair (NER) system is utilized to repair damaged bases. The following is a brief summary of the principal multiprotein NER pathways.

a. UvrABC pathway (short patch repair). As discussed earlier, UV radiation exposure to cells creates three main types of photoproducts: thymine dimers, (6-4) photoproducts, and thymine glycols. In *E. coli*, the first two of these photoproducts are mainly repaired by the nucleotide excision repair pathway. The multienzyme UvrABC excinuclease recognizes and excises the damaged bases via a multi-step process. First, when UV radiation damage occurs to the genome of a cell, several intracellular signals generate increased expression of numerous DNA repair genes. The accumulated evidence indicates that single-stranded DNA is the inducing signal for expression of DNA repair genes (Salles and Defais, 1984; Sassanfar and Roberts, 1990). After exposure to UV radiation induces the formation of thymine dimers, low levels of UvrA (10-20 molecules per cell) detect the helix-distorting effects of thymine dimers and other photoproducts. The UvrA dimerizes and binds to the damaged bases (Fig. 11). UvrB binds to UvrA, and makes an excision on the 3' side of the lesion. UvrC then binds to uvrB, making an excision on the 5' side of the lesion. After the dimerized bases are removed, DNA polymerase I fills in the 12 nucleotide gap left by the NER protein complex, and DNA ligase seals the strand.

The low constitutive levels of UvrA, UvrB and UvrC are able to create these small gaps, resulting in single-stranded regions. The prokaryotic cell is so exquisitely sensitive to formation of these single-stranded regions that it immediately induces elevated expression of RecA. The binding of RecA to single-stranded DNA results in its autophosphorylation which causes it to become active, binding to LexA (a repressor which blocks transcription of several DNA repair genes, notably *uvrA, recA, uvrB, uvrC*, and cleaving it, causing it to fall off the operator of these DNA repair genes (Fig 12). Transcription of these DNA repair genes is turned on and enhanced participation in DNA repair via the UvrABC pathway occurs. RecA appears to be the regulator of the expression of these DNA repair enzymes and when less single stranded DNA is available to activate RecA (which apparently has a short half-life) LexA is able to bind to the operator regions of

Table 5. Substrates for the UvrABC endonuclease of *E. coli.* (Reproduced with permission from Freidberg et al., 1995)

DNA-damaging agent	Adduct(s)
N-acetoxy-2-acetylaminofluorene	*C*-8-Guanine
Anthramycin	*N*-2-Guanine
AP sites	Base loss
AP sites (reduced)	Ring opened AP site
Alkoxamine-modified AP sites	AP site analog
Benzo[*a*]pyrenediolepoxide	*N*-2-Guanine
CC-1065	*N*-3-Adenine
Cisplatin and transplatin	*N*-7-Guanine
Cyclohexylcarbodiimide	Unpaired G and T residues
Ditercalanium	Noncovalent bisintercalator
Doxorubicin and AD32	Intercalated compounds
N-hydroxyaminofluorene	*C*-8-Guanine
N,N'-bis(2-chloroethyl)-*N*-nitrosourea	Bifunctional alkylation
N-methyl-*N'*-nitro-*N*-nitrosoguanidine	O^6-Methylguanine
Mitomycin	*N*-7-Guanine
Nitrogen mustard	Bifunctional alkylation
4-Nitroquinoline-1-oxide	*C*-8, *N*-2-Guanine *N*-6-Adenine
6-4 Photoproduct	*C*-6, *C*-4-PyC
Psoralen	*C*-5, *C*-6-Thymine
Pyrimidine dimer	*C*-5, *C*-6-Pyrimidine
Thymine glycol	*C*-5, *C*-6-Thymine

Table 6. Induction ratios of the different SOS genes *in vivo*. Induction ratios were determined at two different growth temperatures by determination of β-galactosidase activity (Reproduced with permission from Freidberg et al.,1995).

	Induction ratio[a] at:	
Gene	32° C	42°C
lexA⁻	6.7	4.8
recA⁻	11	13
sulA⁻	110	140
umuD⁻*C*⁻	28	17
uvrA⁻	3.4	6.2
uvrB⁻	3.6	3.7
uvrD⁻	4.6	7.2
polB⁻ (*dinA*⁻)	5.2	9.3
dinB⁻	7.3	9.5
dinD⁻	6.2	5.8

these genes and repress their expression. In *E. coli*, induction of RecA is a rapid process and occurs immediately after UV exposure. In *D. radiodurans*, the induction of recA is as immediate but appears to be maximally induced for a longer period of time, perhaps due to the greater amounts of UV radiation damage than *E. coli*.

As mentioned, UvrA in *E. coli* recognizes helical distortions induced by chemical agents as well as UV radiation. It also is capable of recognizing non-helical distortions as well by methods that are currently unknown. Now we know that the gene which complements *D. radiodurans* strain 302, is a component of the UvrABC protein complex. Until the gene was identified (this work) little was known except that the gene responsible for this mutation was capable of participating in the repair of a wide range of helix distorting as well as non-helix distorting DNA damage (Evans and Moseley, 1985). Some of the types of DNA damage *E. coli* UvrA is capable of recognizing is seen in Table 5. The *D. radiodurans* equivalent enzyme is capable of recognizing the same types of damage (Table 5).

Another important difference between *E. coli* UvrA and the equivalent enzyme in *D. radiodurans* is the inducibility of *E. coli* UvrA. *E. coli* UvrA exists at a constitutive level of about 10-20 molecules per cell, and can be induced to a maximum of about 200 molecules per cell. This degree of induction is relatively weak in comparison with the induction levels of other DNA repair enzymes (Table 6). In contrast, *D. radiodurans* UvrA is not induced, remaining constitutively expressed in the cell even after high levels of UV radiation (Evans et al., 1985).

b. SOS Error-Prone Repair and Postreplication Repair. An interesting phenomena can occur when UV-induced thymine dimers are encountered by the replication fork during the exponential growth phase. In *E. coli*, the replication machinery apparently can skip over DNA damage, leaving a large gap about 1,000 bp long to be repaired by the DNA repair proteins. What often happens is that this gap is repaired by recombination with

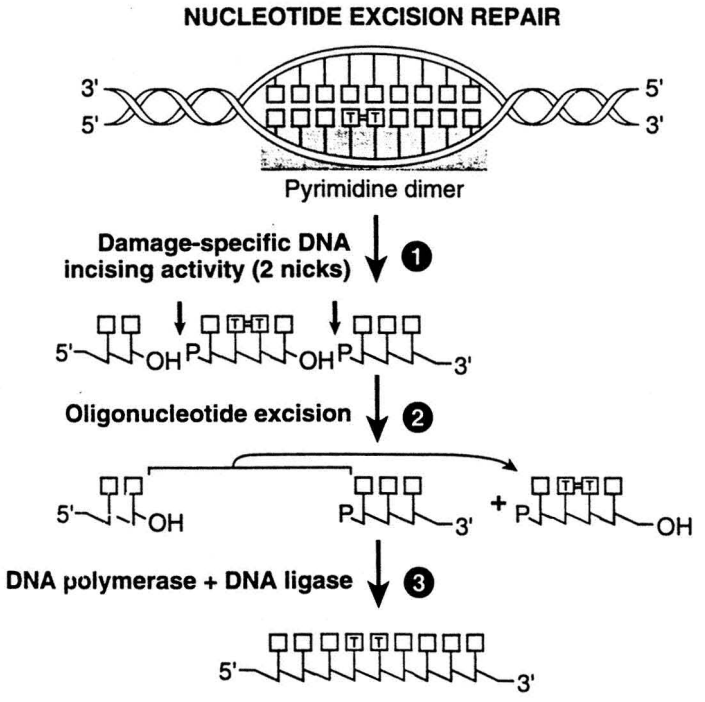


Figure 11. Schematic representation of nucleotide excision repair. (Reproduced with permission from Freidberg et al., 1995)

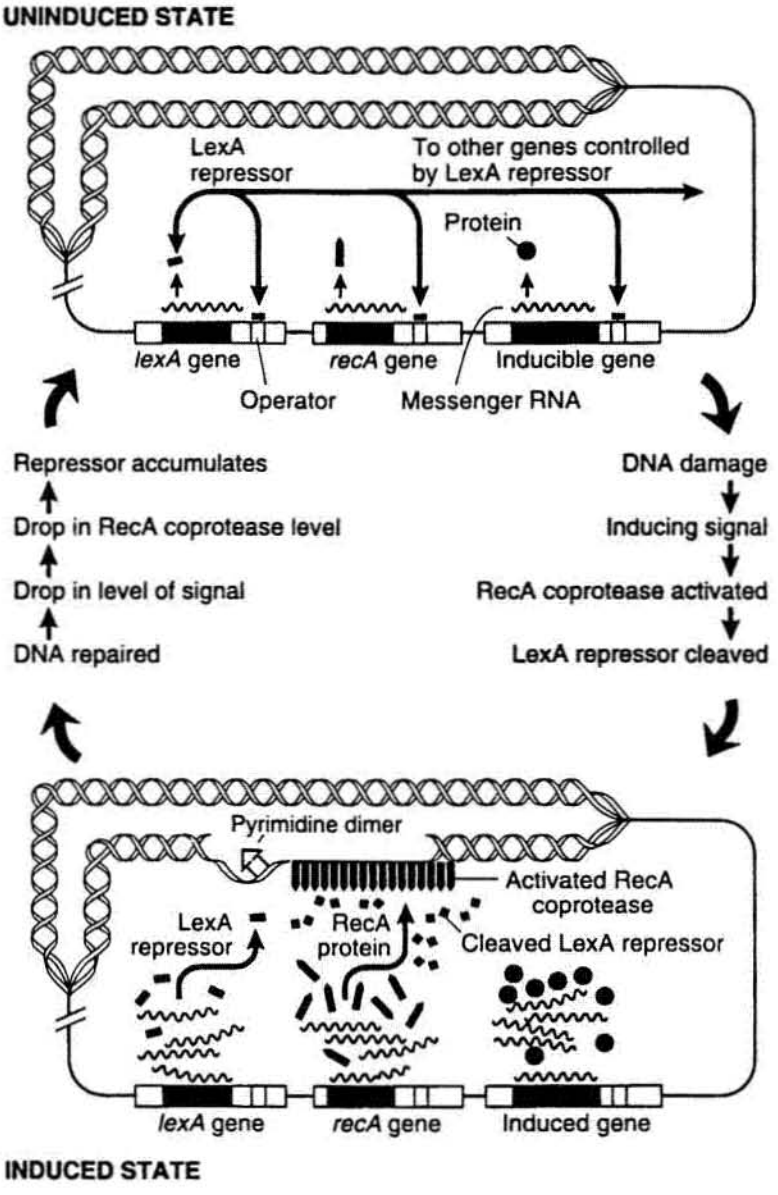


Figure 12. Diagrammatic representation of the mechanism by which the lexA-recA regulon is regulated. (Reproduced with permission from Freidberg et al., 1995)

a daughter strand. This type of repair is called postreplication repair. Error prone repair is due to translesional synthesis. In *E. coli,* a random base is inserted opposite the damaged base, resulting in mutagenic repair (Friedberg et al., 1995). Several independent studies in *D. radiodurans* have determined that repair of DNA damage is completely error-free (Sweet and Moseley, 1974; Kerszman, G., 1975). In fact, it appears that all highly radiation resistant bacteria lack error-prone repair of UV-induced radiation damage (Tempest, 1982).

Several hypotheses have been proposed to explain the existence of error-free repair in *D. radiodurans*. One hypothesis is that *D. radiodurans* does not create the 1,000 bp gaps found in *E. coli* near a thymine dimer, thereby minimizing the probability of two overlapping gaps being present together (Iyer and Rupp, 1971). A second hypothesis is that *D. radiodurans* uses its multiple chromosomes in a highly efficient manner, and the likelihood of a thymine dimer being opposite a gap is very low when a minimum of 8 copies of the same gene exists in every cell in *D. radiodurans* (Moseley and Evans, 1981). To obtain damage to all similar genes at the same spot would require such a high level of UV that the cell would be more likely to die from a lethal hit at a non-DNA target (Setlow and Boling, 1965).

c. Excision plus Recombination Repair. Nucleotide excision repair of cross-links is a special type of DNA repair that requires recombination as an essential part of the repair process. When a cross-link is encountered, such as after MTC treatment, the DNA is excised on the 5' and 3' side of the lesion (Fig. 14). Then recombination repair occurs between strands (Sladek et al., 1988; Sancar et al., 1988; Cole, 1973; Chen et al., 1988; Chen et al., 1988), and the opposite strand is excised and the subsequent gap filled in (Cole, 1973). It is believed that this same process of repair occurs in *D. radiodurans* (Moseley, 1983). In fact, the rec30 mutant of *D. radiodurans* is extremely sensitive to MTC damage, unable to repair even one cross-link per genome (Gutman et al., 1994).

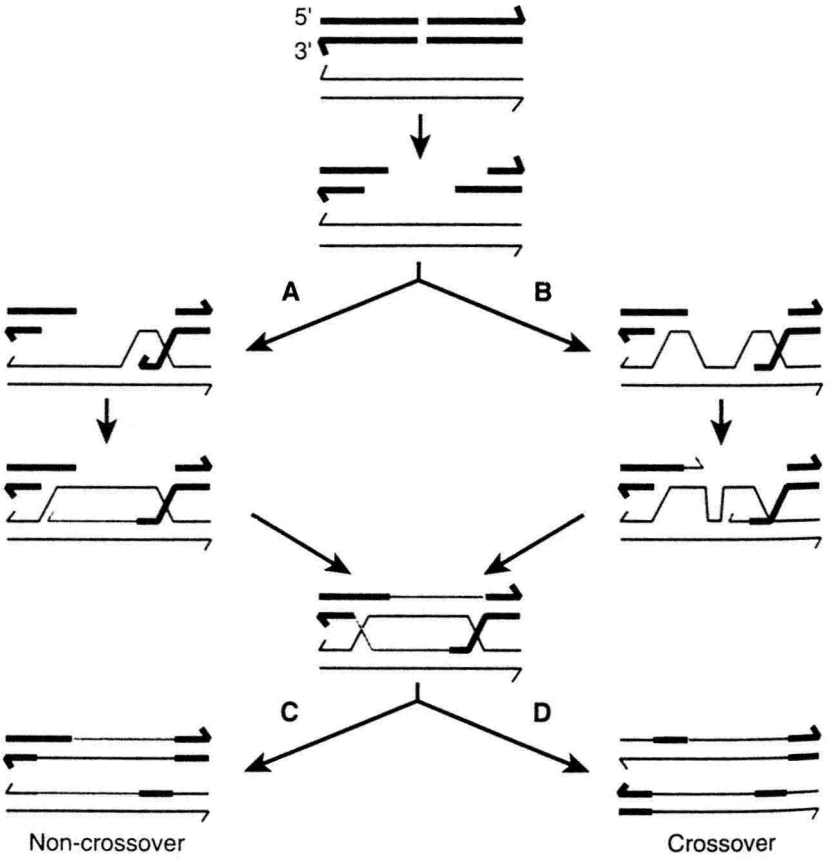


Figure 13. Model for the repair of double-strand breaks. (Reproduced with permission from Freidberg et al., 1995)

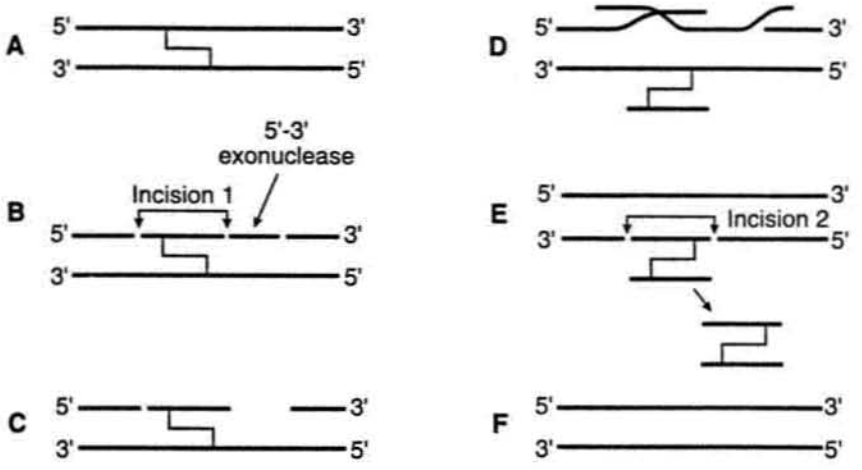


Figure 14. Model for the repair of DNA interstrand cross-links. (Reproduced with permission from Freidberg et al., 1995)

5. Ionizing Radiation Repair

As previously mentioned, ionizing radiation causes cellular damage resulting in single and double strand breaks, thymine glycols as well as free radical production and damage to other non-DNA molecules. Base damage is most often repaired by specific enzymes which recognize each type of DNA damage (e.g. thymine glycols repaired by TG-DNA glycosylase). The repair of single and double strand breaks is not understood at the same level of detail as nucleotide excision repair. However, it seems clear that the repair of double-strand breaks in *E. coli* exposed to ionizing radiation, mitomycin, or UV radiation requires a functional recA$^+$ gene (Krasin et al., 1977; Krasin et al., 1981; Sargentini et al., 1986; Wang et al., 1986). It is known that recA catalyzes strand invasion of a homologous chromosome, and plays a role in branch migration of the invading strand (Fig. 14). In addition, in vitro experiments have shown that the recA protein specifically promotes strand exchange across double-strand breaks (West et al., 1984). In wild-type *E. coli*, the repair of double-strand breaks is also dependent on recB$^+$ and recC$^+$ function (Smith et al., 1987; Smith et al., 1989; Wang et al., 1986).

Essentially, the process of DNA repair is as follows: RecBCD enzyme enters the DNA helix at the double-strand break. It then travels along the DNA, unwinding and rewinding the DNA as it goes. When the enzyme encounters a properly oriented chi site (5' GCTGGTGG-3'), it cuts one DNA strand to produce an invasive single-stranded tail with its 3' end a few nucleotides 3' of the chi site (upstream relative to the direction of travel of the RecBCD enzyme). These tails then serve to invade the homologous intact duplex in a reaction mediated by RecA (Dixon et al., 1991).

The requirement for RecBCD function in the repair of double-strand breaks in wild-type *E. coli* appears absolute: a single double-strand DNA break in the chromosome is lethal in the absence of RecA protein or the RecBCD enzyme, but does not impair growth in the presence of these two proteins (Murialdo, 1988). In *D. radiodurans*, the recA homolog is absolutely required for the repair of double-strand break damage after ionizing

radiation (Moseley and Copeland, 1975). At the present time, no recBCD has been identified in *D. radiodurans*.

D. DNA Repair Deficient Strains in *D. radiodurans*

Radiation-sensitive mutants in *D. radiodurans* have only been successfully isolated via the use of MNNG (Sweet and Moseley, 1974, 1976) or N-methyl-N-nitroso-urea (MNU) (Tempest, 1978), since these are the only known chemical compounds that mutagenize wild-type *D. radiodurans*. Both MNNG and MMU cause base substitution and frameshift mutations in the DNA sequence. Chemical mutagenesis was used to obtain from wild-type, mutant strain, 302, which itself is about 50 times more sensitive to MNNG mutagenesis than the wild-type strain (Tempest and Moseley, 1978, 1980). The 302 strain was used for the development of all subsequent mutant strains due to its ease of mutability.

1. Recombination Mutant rec30

A recombination mutant, rec30, was generated by Moseley and Copeland (1975) via MNNG mutagenesis. This mutant was called a recombination mutant because this strain was found to be defective in natural transformation, a process that requires recombination. With this strain, the efficiency of transformation by homologous chromosomal DNA is reduced in rec30 by 100-fold compared to the wild-type (Masters et al., 1991). This strain is also extremely sensitive to various forms of DNA damage including UV, ionizing radiation and mitomycin-C exposure (Moseley and Copeland, 1975; Masters et al., 1991). The gene which restores rec30's defective repair capacities to wild-type levels was determined to be the homolog of *E. coli* recA (Gutman et al., 1994). The recombination genes for RecB,C,D have not yet been identified in *D. radiodurans*.

2. DNA polymerase I mutant

Recently, it has been determined that two strains derived by chemical mutagenesis, 303 and UV17 are both defective at a locus that encodes a DNA polymerase (Gutman et al., 1993). Both of these strains are highly sensitive to ionizing and UV radiation and MTC (Moseley, 1967; Moseley and Copeland, 1978). This *D. radiodurans* polymerase shares a great deal of sequence homology with DNA Pol I (*polA*) of *E. coli* (Gutman et al., 1993), and it has been determined that the E. coli DNA pol I is just as effective at restoring wild-type phenotype to Deinococcus *polA* mutants as the deinococcal enzyme itself. The DNA Pol I is necessary for the marked DNA-damage resistance of *D. radiodurans* but not sufficient (Gutman et al., 1994).

3. UV Endonuclease-α and -β Mutants

D. radiodurans possesses two independent excision repair pathways, either of which are capable of confering wild-type resistance to UV radiation. One of these pathways, referred to as UV-endonuclease-α pathway, appears to operate by nucleotide excision repair (Al-Bakri et al., 1985, Evans and Moseley, 1983; Moseley and Evans, 1983). This pathway can repair not only pyrimidine dimers, but also mitomycin-induced DNA crosslinks and bulky adducts caused by various chemicals. Until this work, it was believed that UV-endonuclease-α was comprised of proteins encoded by two separate genes, *mtcA* and *mtcB*. It has now been determined in this work that *mtcA* and *mtcB* actually encode a single gene, the homolog of *E. coli uvrA*. It is now believed that the UvrA protein of *D. radiodurans* participates in a pathway analogous to the UvrABC-mediated pathway of *E. coli*. The UvrB and UvrC homologs have not been identified in *D. radiodurans*.

In contrast to what is observed in *E. coli*, UvrA mutants of *D. radiodurans* exhibit wild-type levels of resistance to UV radiation and are able to remove pyrimidine dimers through a second enzyme referred to as UV-endonuclease-β, which requires the gene(s)

uvsCDE. Interestingly, mutants defective in both endonucleases (e.g., *mtcA uvsE* double mutants) are highly sensitive to UV radiation, and are incapable of incising UV-irradiated DNA (Evans and Moseley, 1983; Moseley and Evans, 1983).

Since UV-endonuclease-β only recognizes UV-induced DNA damage, this enzyme may consist of a pyrimidine dimer (PD)-DNA glycosylase, similar to the phage T4 and *M. luteus* PD-DNA glycosylases. UV-endonuclease-β has been partially purified and has a molecular mass of about 36 kDa, and possesses a unique requirement for Mn^+ ions (Evans and Moseley, 1985). Unlike T4 and *M. luteus* PD-DNA glycosylases, UV-endonuclease-β is inactive in the presence of EDTA. In addition, the *D. radiodurans* UV-endonuclease-β activity confers wild-type resistance to UV radiation in *uvrA* mutants. In contrast, *M. luteus uvrA* mutants are only marginally corrected by the gene which encodes the endogenous PD-DNA glycosylase (Nakayama et al., 1992). These and other observations (Gutman et al., 1991) have suggested that UV endonuclease-β might also recognize (6-4) photoproducts in DNA, which accounts for 17% of the photoproducts observed after UV exposure. At the present time, more work needs to be done in this area to reveal the true identity of UV-endonuclease-β.

4. IRS18

Recently, 49 putative **i**onizing **r**adiation **s**ensitive (IRS) strains were isolated by MNNG mutagenesis of strain 302 (Udupa et al., 1994) (Fig. 39). In a subsequent work these strains were further characterized and found to fall into 16 different linkage groups (A-P) (Mattimore et al., 1995). The strains in each of these groups cannot restore radioresistance to each other but do restore resistance to all other recombination-proficient IRS strains. Strain IRS18 falls into a linkage group by itself. Recently it was determined that pHA15 restored ionizing radiation resistance to strain IRS18 (John Batistta; personal communication). Work described in this dissertation focuses on determining the gene responsible for restoring ionizing radiation resistance to the mutant strain IRS18.

E. Specific Aims

Nucleotide excision repair in *D. radiodurans* appears to involve multiple pathways (e. g. UV-endonuclease-α and -β pathway). To date, only two genes involved in this process, DNA Pol I and RecA, have been identified. The isolation and characterization of the other genes involved in this process would contribute to a greater understanding of the means by which *D. radiodurans* efficiently repairs DNA damage. Therefore, the major objectives of the research constituting this dissertation were to isolate and sequence the gene(s) responsible for UV endonuclease-α activity in *D. radiodurans*, and to characterize and elucidate the nucleotide excision repair pathway in *D. radiodurans*.

The first specific aim of this project was to isolate and sequence DNA clones which complemented the mitomycin-C sensitive strains 302 and 262. This goal was accomplished by subcloning sequentially smaller fragments which complemented strains 302 and 262. After this gene was cloned, the objective was to express the *E. coli* homolog of this gene in *D. radiodurans* to determine whether it could functionally be substituted for the deinococcal protein product. Various radiation and chemical treatments were utilized to determine the precise functional differences between the two protein products.

The second specific aim of this project was to isolate and clone the gene which complemented the ionizing radiation sensitive strain IRS18. This goal was accomplished by the same methods as above.

MATERIALS AND METHODS

A. Bacterial Strains and Plasmids

The strains of *Deinococcus radiodurans* and *E. coli* used are listed in Table 7 with their relevant genotypes. Bacteria were stored on agar plates at 4°C and subcultured at 4 to 6 week intervals. All bacteria were grown in batch cultures on an orbital shaker at 32°C unless otherwise stated.

B. Media and Buffers

1. Media

The media for the growth of all bacteria are listed below.

TGY medium	Bactotryptone (Difco)	10% (w/v)
	Yeast Extract (Difco)	5% (w/v)
	Glucose	1% (w/v)

(This medium was used for the growth of all *D. radiodurans* strains.)

Luria Bertani (LB) medium	Bactotryptone (Difco)	10% (w/v)
	Yeast Extract (Difco)	5% (w/v)
	NaCl	10% (w/v)

(This medium was used for the growth of all *E. coli* strains.)

Table 7. Bacterial Strains or Plasmids

Strain or plasmid	Description; relevant genotype	Source (reference)
Deinococcus radiodurans		
R1	Wild Type	Anderson et al., 1956
302	(*mtcA*) MNNG-mutagenized strain R1; γ-ray^{R}UV^{S}MMC^{S}	Moseley and Copeland, 1978
310	302ΩpS11; *mtcA*	302 X pS11
311	302ΩpHA100; *mtcA*, *uvrA*$^{+}$	302 X pHA100
262	(mtcB) MNNG-mutagenized strain R1; γ-ray^{R}UV^{S}MMC^{S}	Moseley and Copeland, 1978
270	262ΩpS11; *mtcA*	262 X pS11
271	262ΩpHA100; *mtcB*,*uvrA*$^{+}$	262 X pHA100
IRS18	(*irrB*,*mtcA*) MNNG-mutagenized strain 302; γ-ray^{S}UV^{R}MMC^{S}	Gupta and Battista, J. 1994
IRS181	(irrB^{+},mtcA^{+}) IRS18ΩpUE58; 5.6 kb *D. radiodurans* chromosomal fragment containing *mtcA* and *irrB* genes	Gupta and Battista, J. 1994
Escherichia coli		
DH5α	*uvrA*$^{+}$*recA*	Life Technologies
JL106	*uvrA*	Barbara Bachman
Plasmids		

pUE50	cosmid pJBFH *BamH*I::37.9kb *D. radiodurans BamH*I chromosomal fragment containing *mtcA* and *mtcB* genes	Al-Bakri et al., 1985
pS11	aphA (Km^R)12.5 *EcoR* I fragment of *D. radiodurans* chromosomal DNA in pMK20	Smith et al., 1988
pHA100	pS11 *Bgl* II-*Dra*I::*BamH*I-*Hind*III fragment from pSST10 containing *E. coli uvrA* complete coding sequence	This work (pSST10 from Oleg Kovalsky)
pHA15	pBS (SK+) *EcoR*I::5.6 kb *D. radiodurans EcoR*I chromosomal fragment containing *mtcA* gene	This work
pUE58	pAT153 *EcoR*I::5.6 kb *D. radiodurans EcoI* chromosomal fragment containing *mtcA* and *irrB* genes	Al-Bakri et al., 1985
pHA15b	pBS (SK+) Acc1::3.0 kb *D. radiodurans Acc*I chromosomal fragment containing *mtcA* gene (blunt-end ligation); pHA15 cut with *Acc*I, filled-in then religated. *Acc*I site is destroyed.	This work
pHA15b.1	pBC *Xho*I:: 1.1 kb *D. radiodurans Xho*I chromosomal fragment containing *mtcA* and *irrB* genes	This work
pHA15b.2	pBC *Xho*I::1.52 kb *D. radiodurans Xho*I chromosomal fragment containing 1.0 kb ORF	This work
pHA18	pBS (SK+) *EcoR*I::316bp *D. radiodurans EcoR*I chromosomal fragment containing *uvrA* sequence isolated from cosmid pUE502	This work
pHA16	pBS (SK+) *EcoR*I::2.6 kb *D. radiodurans EcoR*I chromosomal fragment containing *mtcA* gene	This work
pHA17	pBS (SK+) *EcoR*V::1.3 kb *D. radiodurans Sty*I chromosomal fragment containing *mtcA* gene	This work

S.O.C. Medium (Gibco BRL, Gaithersburg, MD)

Tryptone	2% , Yeast Extract	0.5%
NaCl	10 mM, KCl	2.5mM
$MgCl_2$	10 mM, $MgSO_4$	10 mM
Glucose	20 mM	

(This medium was used to obtain maximum transformation efficiency of *E. coli.*

Superbroth To 900mls of deionized H2O, the following was added:

Bacto-tryptone, 12g; Bacto-yeast extract, 24g; and glycerol, 4 ml.

TGY and LB Media were solidified for agar plates with 15 g/l of bacteriological agar (Difco Laboratories, Detroit, MI).

2. Buffers

Routine buffers were purchased from Biofluids, Inc., Rockville, MD. Other sources are described as they arise.

C. DNA Isolation and Analysis

1. Bacterial Genomic DNA Isolation.

Isolation of genomic DNA was performed according to the methods outlined by Ausubel (1987). Essentially *D. radiodurans* strains were grown in 5 mls TGY medium and incubated at 30°C to late exponential phase (which is about 48 hours). Aliquots of these cells were then pelleted in a microcentrifuge and resuspended in 567 µls of TE buffer (Tris 10mM pH 8.0; EDTA 1mM), 15 µls of 20% SDS, and 10 µls of 20 mg/ml proteinase K. The resulting solution was mixed and incubated for 1 hour at 37°C. To the solution was added 100 µls of 5 M NaCl followed by 80 µls CTAB/NaCl solution

(4.1% (w/v) NaCl, 10% (w/v) Hexadecytrimethyl ammonium bromide (CTAB). The mixture was incubated for 10 minutes at 65°C to disrupt the integrity of the cell wall. The liberated chromosomal DNA was extracted with an equal volume of chloroform/isoamyl alcohol (24:1 v/v). The solution was centrifuged for 5 min. in a microcentrifuge to separate aqueous and organic phases. The top layer (aqueous phase) was transferred to a fresh tube and extracted once or twice with phenol/chloroform/isoamyl alcohol (25:24:1 v/v) to remove all protein membrane components. The solution was spun in a microcentrifuge for 5 minutes and the aqueous phase was transferred to a fresh tube. The chromosomal DNA was precipitated with 0.6 volumes isopropanol, by slowly adding the isopropanol on top of the aqueous layer and slowly rocking the eppendorf tube. The chromosomal DNA quickly formed a white string-like precipitate which was removed to a new tube with 70% ethanol using an inoculating loop. The ethanol was removed and the pellet was air dried for 10 minutes or until all the ethanol had evaporated. The resulting pellet was resuspended in 100 µl TE buffer.

2. Bacterial Plasmid DNA Isolation.

Rapid isolation (mini-preparation) and large-scale preparation of plasmid DNA were adapted from the methods outlined by Maniatis (1989). Cells were grown in 5.0 mls (LB) supplemented with appropriate antibiotic selection and incubated overnight at 37°C with vigorous shaking. Aliquots of the overnight cultures were then used for either mini-preparations or inoculated into larger volumes of LB for large-scale plasmid preparations. "Superbroth" which is an enriched derivative of LB supplemented with glucose was often used to enhance the growth of the bacteria.

Mini-preparations of plasmid DNA were performed according to the boiling-lysozyme method. Briefly, 1.5 mls of each culture was transferred to a 1.5 ml eppendorf tube and spun in a microcentrifuge for 2 minutes. The supernatant was discarded and the pellet was resuspended in 350 µls STET buffer [(8.0% (w/v) sucrose, 5.0% (v/v) Triton

X-100, 50mM EDTA, 50 mM Tris (pH 8.0) plus lysozyme (250 mg/ml)]. The tubes were placed in a boiling water bath for 2 minutes, then spun immediately for 15 minutes at room temperature. The supernatant was transferred to new 1.5 ml eppendorf tubes and 300 µls isopropanol was added. Tubes were placed on ice for 5 minutes before pelleting DNA for 15 minutes at 4°C. After discarding alcohol, the pellets were air-dried for not more than 15 minutes. Plasmid DNA was dissolved in 50 µls dH_20 and stored at -20°C until needed.

Large-scale plasmid preparations were performed using the alkaline lysis method (Birnboim and Doly, 1979) as described by Maniatis (1989). Purification of closed circular DNA was achieved by centrifugation in cesium chloride (CsCl)-ethidium bromide density gradients. Plasmid DNA was then phenol/chloroform (50:50 v/v) extracted, ethanol precipitated, resuspended in 50-100 µl of TE and stored at -70°C. Quantity and purity of DNA was determined spectrophotometrically by monitoring at wavelengths of 260 nm and 280 nm.

3. Restriction Enzyme Analysis.

Enzymes and buffers used in the various cloning procedures were purchased from either New England Biolabs, Inc. (Beverly, MA), or Bethesda Research Laboratories (Gaithersburg, MD) and used according to manufacturers' instructions. Briefly, plasmid DNA was diluted in buffers supplied by the manufacturer for restriction enzyme digestions along with sufficient restriction enzyme to allow complete digestion to occur in 2 hours. Following restriction digestion, reactions were stopped by the addition of 10X loading buffer (0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol FF, 30% glycerol in water) and loaded onto an agarose gel if no further manipulation of the DNA was required. In the event further manipulation was required, the DNA was subjected to phenol/chloroform extraction followed by ethanol precipitation and resuspension in approximately 25 µl TE or water for use in subsequent reactions. If two or more DNA fragments resulted from restriction enzyme digestion, individual fragments

were separated by agarose gel electrophoresis as described (see Agarose Gel Electrophoresis). DNA fragments were recovered by excision of the desired fragment(s) from the gel with a clean razor blade while illuminating the gel with long wavelength ultraviolet (UV) light. The fragment(s) were then extracted from the agarose slice either by spinning the DNA-containing agarose slice in an ultracentrifuge at 50,000 rpm for 15-30 minutes at room temperature and recovering the DNA containing supernatant or by using the Qiaex DNA extraction kit (Qiagen Inc., Chatsworth, CA) as specified by the manufacturer's protocol. The isolated DNA was resuspended in a final volume of 20 µl TE or water, and stored at -20°C until needed for cloning.

D. Molecular Cloning of DNA Fragments

1. Construction of Recombinant DNA Molecules.

The strategy for generation of recombinant plasmids varied depending on the particular construct desired. In most cases, restriction enzyme digestion resulted in a DNA fragment possessing 5' termini that were compatible with unique restriction sites within a cloning vector (i.e. pBluescript® II SK, pBC SK). If DNA fragments containing 5' protruding ends were to be cloned into a blunt-end restriction site in the cloning vector, then the 5' protruding ends of the DNA fragments were "filled-in" in order to make them compatible with the vector restriction site. A typical "fill-in" reaction was performed immediately after a restriction digestion using the same buffer. To the restriction digestion was added 1 µl dNTPs (2 mM each dNTP), 1 µl T4 DNA polymerase (1-5 Units) in a total volume of 20 µls. The reaction was incubated for 30 minutes at room temperature, stopped by the addition of 1 µl 500 mM EDTA, and phenol/chloroform extracted twice to remove proteins. The DNA was precipitated using 95% ethanol and 0.3 M sodium acetate, and the resulting pellet resuspended in 20 µl TE.

Dephosphorylation of vector DNA termini was performed in order to suppress self-ligation. The dephosphorylation reaction was usually performed at the end of a restriction digestion. To a typical 20 µl restriction digestion 1 unit of calf intestinal phosphatase (CIP), was added and dephosphorylation of exposed vector ends was allowed to occur for 30 minutes. The reaction was then incubated at 60°C for 10 minutes to inactivate the phosphatase and ensure that the enzyme released the vector DNA. In the event that inactivation of the phosphatase was not complete the protein would stay bound to the DNA and upon subsequent extraction with phenol/chloroform the vector DNA would be pulled into the organic phase and result in low yields of vector DNA. In order to circumvent this problem longer incubation times at 60°C were performed or shrimp alkaline phosphatase (which does not bind tightly to DNA) was occasionally used. After inactivation of the phosphatase the DNA was extracted with phenol/chloroform followed by ethanol precipitation and was resuspended in a final volume of 20 µl TE. Prior to ligation reactions, a small amount of both fragment and vector DNA were analyzed by agarose gel electrophoresis as described below to confirm integrity and determine approximate concentration.

Typical ligation reactions included 80-100 ng prepared vector DNA, varying amounts of DNA fragment insert ranging from 1:2-1:20 molar ratio (vector:insert), 1 µl ligase buffer, 1 µl ligase (5-200 Units), and water in a total volume of 10 µl which was incubated overnight at 12-15°C.

2. Bacterial Transformation.

a. *E. coli* Transformation.

Competent DH5α cells were purchased from Gibco, BRL (Grand Island, New York), and transformations were performed according to manufacturer's recommendations. Competency of the bacteria was tested by transformation with a closed circular plasmid (e.g., pUC19) to determine transformation efficiency. In brief, a 10 µl

ligation reaction was diluted in 100 µls competent cells and incubated on ice for 30 minutes. The cells were then subjected to heat shock at 37°C for two minutes and immediately cooled on ice for two minutes. The 100 µl transformation mix was then added to 900 µl S.O.C. medium and incubated at 37°C for 1 hour. An aliquot of the culture (100 µl) was spread on LB/agar plates supplemented with the appropriate antibiotic (usually ampicillin [amp, 50-100 µg/ml]) and incubated overnight at 37°C. For α-complementation screening, agar plates were supplemented with 2% (w/v) 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) (Gibco, BRL, Grand Island, New York) and 25 mg/ml isopropylthio-β-D-galactoside (IPTG) (Sigma Chemical Co., St. Louis, MO). On these plates, bacteria which are β-galactoside positive (*lac*Z$^+$) appear blue while those that are negative (*lac*Z) appear white. The white colonies indicate that the DNA fragments have been successfully incorporated into the polycloning site of a cloning vector (e.g., pBS, or pBC), and has disrupted the amino terminal end of β-galactosidase.

b. *D. radiodurans* Transformation.

D. radiodurans cultures were routinely made competent by the procedure developed by Tirgari and Moseley (1980). Cultures (5 mls) were grown for 18 hours at 30°C. The 5 ml culture was then inoculated into 50 mls fresh TGY broth and incubated in a baffled 250 ml flask for 3 hours or until an OD_{600} of 0.5 was obtained. The cells were pelleted at 3K rpm for 10 min. at 4°C. The pellet was resuspended in 5 mls freshly prepared TGY containing 100 mM $CaCl_2$ and 10% glycerol. The suspension was divided into 200 µl aliquots, then immediately frozen at -70°C.

Only competent cells less than 2 months old were used for transformation. Competent cells were thawed on ice. To chilled 50 ml Corning tubes were added 100 µls cells, and 1-5 µg DNA. The solution was mixed gently and placed on ice for 10 min. The suspension was then placed at 32°C for 30 min. with gentle shaking. To the transformation mix was added 900 µls TGY and incubated overnight at 32°C with vigorous shaking.

After 18 hours incubation 100 µls of transformation mix was spread on the appropriate selective agar.

3. Identification of Recombinant Plasmids.

Bacterial colonies containing recombinant plasmids were identified by restriction analysis of plasmid DNA as described by Maniatis (1989). In cases where insertion of the desired DNA fragment resulted in inactivation of the β-galactosidase gene in the vector (white colonies), restriction analysis of plasmid DNA for several transformants was performed.

4. Identification of Recombinant Chromosomes.

All DNA fragments destined for chromosomal integration were cloned into the replication-origin-lacking, chromosomal integration vector pS11 and identified via selection on kanamycin (10 µg/ml) containing TGY agar plates.

E. Sequencing of Double-stranded DNA by the Dideoxy-sequencing Method.

Double-stranded DNA sequencing was performed according to manufacturer's protocol (United States Biochemical, Cleveland, Ohio). Double-stranded DNA template was denatured prior to sequencing in one of two ways. DNA was denatured by the alkaline-denaturation method (Maniatis, 1989), or by the spin-dialysis method. In the latter method 5-10 µgs DNA in a 15 µl volume was denatured by the addition of 4 µls NaOH, 1mM EDTA, and left at room temperature for 5 minutes followed by spin-dialysis. To a 0.5 µl eppendorf tube (with a hole punched into the bottom of the tube by a 21 gauge needle) were added 200 micron glass beads (Gibco BRL, Gaithersburg, MD) in water

followed by 300 µls Sepharose CL-6B (Gibco BRL, Gaithersburg, MD). The slurry of beads and sepharose was centrifuged for 4 minutes at 200g after which the 0.5 ml tube was transferred to an intact 1.5 ml tube, the DNA sample added, and the tubes were spun as above. The denatured single-stranded DNA passed through the Sepharose and glass beads and into the 1.5 ml tube while the non-denatured double-stranded DNA was trapped in the Sepharose. To 8.5 µls of the denatured DNA was then added 1.5 µl 100mM Tris pH 8.0, 50mM $MgCl_2$ and 1 µl primer (10 µg/ml) and incubated at 37°C for 15 minutes.

Briefly, the chain termination DNA sequencing method was performed as follows. After oligonucleotide primers were annealed to the single-stranded DNA template the labeling reaction was performed. To the ice-cold annealed DNA mixture was added 1 µl freshly prepared 0.1 M dithiothreitol (DTT), 2 µls diluted labeling mix (7.5 uM dGTP, dATP, and dTTP), 0.5 µl [^{35}S] dCTP, 1 µl diluted Sequenase Polymerase and 1 µl Klenow fragment DNA Polymerase. The reaction was mixed and incubated at room temperature for 5-15 minutes and then terminated as described below. In this work dCTP was used in all sequencing reactions instead of dATP because *D. radiodurans*' GC:AT ratio is about 68:32. Therefore the greater incorporation of [^{35}S] dCTP gives a stronger signal on X-ray film. Also, the use of Klenow fragment in the labeling reaction decreased probability of secondary structures forming on the DNA template which were a great problem in earlier experiments due to to the high GC content of the DNA. To terminate the labeling reactions 3.5 µls of the labeling reaction was added to each of four termination tubes (G, A, T, and C) which contained 8 µM of a single dideoxynucleotide as well as 80 µls (88 µM) of the other three nucleotides plus 50 mM NaCl. The termination reaction was allowed to proceed for 5 minutes at 37°C. The reaction was stopped by adding 4 µls Stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF). The samples were heated to 75°C, for 2 minutes, then immediately loaded (3.5 µls) onto a polyacrylamide sequencing gel.

F. Preparation of Protein Extracts

Cells were grown in a minimum of 500 mls TGY overnight with vigorous shaking at 32°C. Cells were pelleted and resuspended in 5 mls Tris-HCl (15 mM, pH 8.0) containing 2-mecaptoethanol (2 mM) and passed three times through a French pressure cell at 10,000 psi at 4°C. In earlier experiments cells were also sonicated for 10-15 minutes to release intracellular proteins; however, upon examination of antibody reactions on Western blots it was determined that during the sonication procedure degradation of the proteins of interest was occuring. The French press procedure was found to yield higher quantities of intact intracellular proteins and was subsequently used for all pertinent experiments in this work. Protein content of the crude extract was assayed using a Protein Assay Kit (BioRad Laboratories, Melville, New York).

G. Gel Electrophoresis

1. Agarose Gel Electrophoresis.

DNA fragments greater than 0.5 kilobase (kb) in size were separated using horizontal 0.8-1.0% (w/v) agarose slab gels in 1x TBE running buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0). DNA samples containing 1x loading buffer (0.25% [w/v] bromophenol blue, 0.25% [w/v] xylene cyanol FF, 30% glycerol) and 0.5 µg/ml ethidium bromide were electrophoresed at constant voltage. The DNA was visualized by placing the gel directly on a UV light box (310 nm) and photographed using Polaroid type 55 or 57 film with a Polaroid MP-4 camera.

2. SDS-Polyacrylamide Gel Electrophoresis.

a. Separation and Detection of DNA.

DNA fragments were also separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli

(1970). Standard gels (20 cm x 40 cm) were run on a BRL sequencing gel apparatus (Gibco BRL, Grand Rapids, MI), prepared with a gel concentration of 8% acrylamide/bis-acrylamide. A stock solution of 10% (w/v) ammonium persulfate (APS) in water along with N,N,N'N'-tetramethylethylenediamine (TEMED) was used to catalyze the polymerization of the acrylamide. The running buffer consisted of 1x TBE (10.8% Tris base, 0.55% boric acid, and 0.93% $Na_2EDTA\text{-}2H_20$).

DNA samples diluted with Stop buffer (95% formamide, 20mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF) were heated to 75°C for 2 minutes to denature the DNA prior to loading. Sequencing gels were run at a constant current of 60 mAmps until the dye reached the bottom of the gel (which was usually about 2.5 hours). Often a second loading of the samples was done in order to get the longest readable stretch of bases. The most visible sequences were between the range of 50-200 base pairs. At the end of a run, the sequencing apparatus was dissassembled with the gel still attached to the longest plate. A large sheet of Whatman blotting paper was pressed flat against the gel and then quickly removed carrying with it the gel. Clear plastic wrap was placed on top of the gel and the assembly was immediately placed on a gel drier and dried for 1.5 hours. After the gel was thoroughly dried to the Whatman paper, the plastic wrap was removed and the blot was placed in a film cassette and exposed overnight to Kodak X-OMAT autoradiographic film.

b. Separation of Proteins.

Proteins were separated by one dimensional denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). Minigels were run on a Mini-Protean II gel rig (Bio-Rad Laboratories, Melville, NY), and prepared from a 30% (w/v) acrylamide stock containing 29.2% (w/v) acrylamide and 0.8% (w/v) N,N'-methylene-bisacrylamide (bis) in distilled water. The lower gel buffer (LGB) consisted of 0.1% (w/v) SDS in 375 mM Tris-HCl (pH 8.8) and the upper gel buffer (UGB) contained 0.1% (w/v) SDS in 125 mM Tris-HCl (pH 6.8). A stock solution of 10% (w/v) ammonium persulfate (APS) in water along with N,N,N',N'-tetramethylethylenediamine (TEMED) was used to catalyze the polymerization of the acrylamide. Running buffer consisted of 25 mM Tris, 192 mM glycine, and 0.3 mM SDS (pH 8.8). The separating gel was usually 10% (w/v) acrylamide in LGB with 3.75 µl/ml APS and 0.75 µl/ml TEMED. The stacking gel consisted of 4.5% (w/v) acrylamide in UGB with 5 µl/ml APS and 2.5 µl/ml TEMED.

Protein samples diluted with sample buffer (2% [w/v] SDS, 10% [v/v] glycerol, 10% 2-mercaptoethanol, 50 mM Tris [pH 6.8], 100 mM dithiothreitol (DTT), 0.1% bromophenol blue) were boiled for ten minutes to solubilize proteins prior to loading. At least one well per gel was loaded with prestained high molecular weight protein standards (B.R.L., Gaithersburg, MD). Minigels were run at a constant 200 volts (V) until the dye front reached the bottom of the gel. At the end of a run, rigs were disassembled and gels were transferred to nitrocellulose for immunoblot analysis.

H. Detection of DNA on Agarose Gels

If specific DNA bands needed to be identified then the agarose gels were transferred to nitrocellulose and radiolabelled DNA probes were employed. A DNA agarose gel would be submerged in denaturation solution (1.5M NaCl, 0.5M NaOH) to

denature the double stranded DNA in the agarose. Then the gel was placed in neutralization solution (1.5M NaCl, 1.0M Tris, pH7.0). The gel was then placed in a horizontal electrophoresis tank on top of a sheet of nitrocellulose with several layers of Whatman paper as well as paper towels placed above it. The gel tank was filled with 20x SSPE (17.53% NaCl, 0.276% NaH_2PO_4, 0.0074% EDTA, pH 7.4; Biofluids Inc., Rockville, MD). A large object was placed on top of the assembly to compact it, and transfer of the single-stranded DNA fragments occurred by capillary action overnight. When transfer of the DNA was complete the nitrocellulose was cross-linked for 5 minutes in a DNA Transfer Linker (Fotodyne; New Berlin, WI) and then baked at 60°C in a vacuum oven for 1 hour. The dried blot was then stored until needed or immediately placed in pre-hybridization solution (8% 20x SSPE, 0.8% SDS, 4% dry milk powder, 0.001% NaN_3, 2% (w/v) denatured salmon sperm DNA) and incubated at 65°C for 3 hours. The pre-hybridization solution was then discarded and a hybridization solution (6% 20x SSPE, 1.6% SDS, 10% dextran sulphate, plus DNA probe was added to the blot and incubated at 65°C overnight. The DNA probe (pS11::uvrA) was labeled via the random primer labeling method. The hybridization solution was discarded and the blot was incubated in wash buffer (1% 20x SSPE, 1% SDS) at room temperature for 30 minutes. Wash buffer was changed three times with the final wash at 65°C. The nitrocellulose filter was immediately blotted between two Whatman paper filters to remove excess buffer and wrapped in clear plastic wrap and placed in a film cassette and exposed to Kodak XAR autoradiographic film overnight.

I. Detection of Proteins on SDS-Polyacrylamide Gels

1. Coomassie Staining.

Proteins separated on SDS-polyacrylamide gels were stained in a Coomassie brilliant blue solution (0.2% (w/v) Coomassie blue R-250, 30% (v/v)

isopropanol, and 20% glacial acetic acid). The gel was immersed in staining solution for 15-30 minutes at room temperature with rocking, followed by immersion in destain solution (10% (v/v) acetic acid, 5-10% (v/v) methanol) for at least 1 hour or until protein bands were clearly visible.

2. Immunoblotting.

To identify proteins separated by SDS-polyacrylamide gels, proteins were transferred to Immobilon PVDF transfer membrane (Millipore Corp., Bulington , MA). The Immobilon transfer membrane is a polyvinylidene difluoride microporous membrane that is ideal for protein blotting. This hydrophobic membrane has a high protein binding capacity and high mechanical strength. It also shows high resistance to methanol, a solvent used in many blotting and staining protocols that causes nitrocellulose membranes to shrink and distort. Transfer of proteins was achieved with a semi-dry blotting apparatus (Bio-Rad Laboratories, Melville, NY) or a Bio-Rad Mini-Transblot II. Prior to transfer, gels were soaked in transfer buffer (192 mM glycine, 25 mM Tris/HCl, 0.01% SDS, 20% (v/v) methanol) and sandwiched next to a methanol wetted nitrocellulose membrane between transfer buffer saturated Whatman 3M filter paper and Whatman blotting paper for assembly in transblot cassettes. Semi-dry transfer was achieved by running at 20 volts for 1 hour. Mini-transblot rigs were filled with transfer buffer and run at 100 volts for one hour with ice-pack cooling. Following transfer, the nitrocellulose membranes were blocked with blocking buffer (1% (v/v) bovine serum albumin, 50 mM Tris/HCl pH 7.46, 150 mM NaCl, 0.05% NaN_3) at 4°C overnight or until ready to probe with antibody.

Typically, blots were probed by incubation with the primary antibody diluted 1:50-2000 in blocking buffer. Blots and antibody were placed in small trays and incubated for eighteen hours with rocking at 4°C followed by washing three times for 20 minutes each at room temperature in TBST (150 mM NaCl, 50 mM Tris/HCl (pH 7.4), 0.05% (v/v) Tween-20). Blots were then incubated with alkaline phosphatase-conjugated secondary

antibody (goat anti-rabbit IgG (when primary antibody was polyclonal); or goat anti-mouse IgG (when the primary antibody was monoclonal); Bio-Rad Laboratories, Melville, NY). TBST buffer without NaN_3 was used for washes following secondary antibody since sodium azide interferes with the substrate-enzyme reaction and results in weak signal on X-ray film. Detection of specific immobilized antigens was determined by enhanced chemiluminescence (ECL™, Amersham, Arlington Heights, IL) western blotting according to the manufacturer's protocol. Briefly, a chemical solution containing luminol and phenols including peracid were mixed and poured onto the blots containing primary antibody and horseradish-peroxidase conjugated secondary antibody. A chemical reaction proceeds in which the peroxide catalyzes the oxidation of the luminol in alkaline solution. Immediately following oxidation, the luminol is in an excited state which then decays to ground state via a light emitting pathway. Enhanced chemiluminescence is achieved by addition of phenols. Light is emitted as the product is oxidized, thus exposing autoradiographic film. The light produced by this enhanced chemiluminescent reaction peaks after 5-20 minutes and decays slowly thereafter with a half life of approximately 60 minutes.

J. Antisera

Rabbit polyclonal antisera directed against *E. coli* UvrA was obtained from Dr. Lawrence Grossman (Department of Biochemistry, The Johns Hopkins University). Mouse monoclonal antibody A2A3, which recognizes the region of UvrA between amino acids 680 and 940, was also obtained from Dr. Oleg Kovalsky in the laboratory of Dr. Lawrence Grossman (Department of Biochemistry, The Johns Hopkins University).

K. Treatment with DNA Damaging Agents

D. radiodurans cells were grown for 48 hours with vigorous shaking at 32°C. *D. radiodurans* is capable of sustaining great amounts of DNA damage to it's chromosome and

is capable of repairing this damage in a timely manner. Differences in the repair rate of DNA damage can be seen in cells growing exponentially versus cells in late exponential or stationary phase in the cell cycle. To maintain consistency all cells in this work were exposed to DNA damaging agents during stationary phase.

1. Treatment of DNA with Ionizing Radiation.

From *D. radiodurans* cells which were grown for 48 hours a small sample was taken (1.0 ml) and placed in an eppendorf tube and placed on ice. Cells were immediately exposed to gamma radiation emitted from a Co^{60} source for 30 minutes to 1 hour and 30 minutes. Cells were maintained on ice during exposure time. Immediately following exposure to ionizing radiation the cells were diluted and plated on TGY agar plates and incubated for 3 to 5 days prior to counting of colonies.

2. Treatment of DNA with UV Radiation.

D. radiodurans cells were grown for 48 hours and then serially diluted and plated on TGY agar. The lids of the agar plates were removed and the plates were exposed to UV radiation emitted from a germicidal lamp (254 nm) at a dose rate of 1.0 $J/m^2/s$ resulting in total cell exposure of approximately 50 J/m^2 to 1,500 J/m^2. The lids were immediately placed back on the plates when exposure times were completed and the plates were incubated for 3 to 5 days.

3. Treatment with Mitomycin-C (MMC).

Mitomycin C (Sigma Chemical Corp.), a bifunctional alkylating agent which causes bulky monoadduct lesions as well as diadduct crosslinks between DNA strands, was added to stationary growing cells at a concentration of 1 µg/ml. At thirty minute intervals samples were removed from the incubating cultures and diluted and spread

on TGY agar plates. The plates were incubated at 32°C for 3-5 days and cell survival was calculated.

4. Treatment with Me_3psoralen + NUV Radiation.

D. radiodurans strains were grown two days to ensure that all cells reached stationary phase. Cells were centrifuged and resuspended in dH_20. A 10 µl volume of a saturated ethanol solution of Me_3psoralen was added per milliliter of dH_20. Although the Me_3psoralen was added at a concentration of about 6-7 µg/ml, the concentration of Me_3psoralen dissolved in dH_20 was approximately 0.6 µg/ml (Ashwood-Smith and Grant, 1977). Cells were incubated for 10 minutes at 32°C. The cells were then irradiated (with agitation) to 120 J/m^2 using a 310 nm near-UV lamp. Following irradiation the cells were immediately centrifuged, washed with TGY medium, appropriately diluted, plated on TGY agar plates, and incubated for 3-5 days.

5. Treatment with N-methyl-N-nitro-N-nitrosoguanidine (MNNG).

Five ml samples of stationary cell cultures were centrifuged, and the bacteria were resuspended in half the original volume with phosphate buffer pH 7.0 in which MNNG had been freshly dissolved. The suspensions were shaken at 32°C for up to a maximum of three hours. At thirty minute time intervals a 0.1 ml sample was removed, rapidly centrifuged in a microcentrifuge, washed, and resuspended in an equal volume of TGY medium. The sample was diluted with TGY medium and plated on TGY agar and incubated at 32°C for 3-5 days prior to counting of colonies.

RESULTS

A. Structural Features of *D. radiodurans uvrA*

1. DNA Sequence of the Locus Defective in DNA Damage-sensitive Strains 302 and 262.

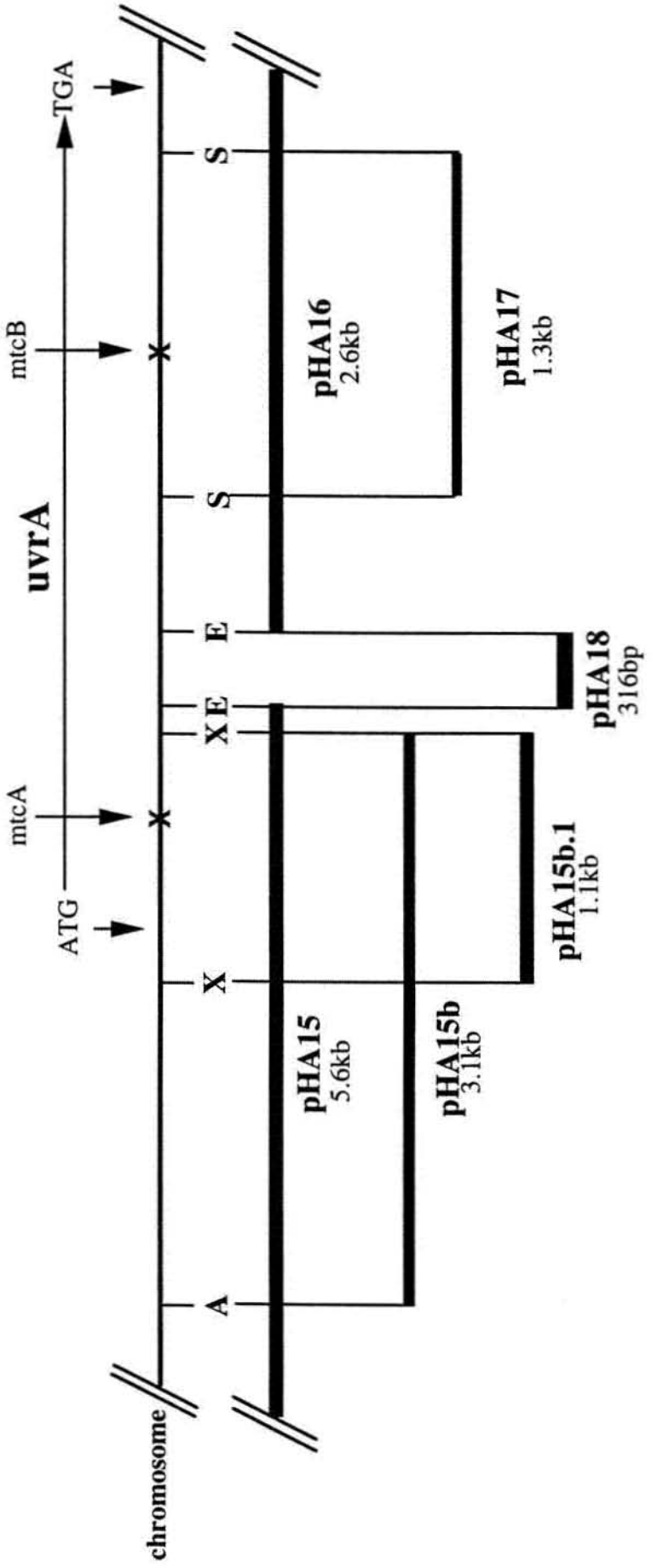
As previously mentioned *D. radiodurans* is naturally transformable and easily recombines homologous donor DNA with its chromosome (Al-Bakri et al., 1985; Tirgari and Moseley, 1980). By exploiting this function, identification of the defective loci in strains 302 and 262 proceeded by determining which particular DNA clones from a cosmid library of wild-type *D. radiodurans* transformed these mutant strains to wild-type mitomycin C resistance (MMC^R) (Al-Bakri et al., 1985).

One cosmid clone, pUE502 (Al-Bakri et al., 1985) was found to transform both 302 and 262 to wild-type MMC resistance as determined by colony formation on TGY agar plates containing 0.05 ug MMC. This cosmid clone was digested with *EcoR* I and the resulting five fragments were subcloned. One subclone, pUE58, a 5.6 kb *EcoR* I fragment, complemented strain 302, restoring MTC^R (Fig. 15). This 5.6 kb fragment was initially cloned into a pBR322 derived vector (Al-Bakri et al., 1985), but in this work was subcloned into pBS SK+ (*EcoR* I site) and renamed pHA15.

Another subclone, pUE59, a 2.6 kb *EcoR* I fragment, complemented strain 262, restoring MTC resistance. This clone, pUE59, was also subcloned into pBS SK+ (*EcoR* I) and renamed pHA16. These two clones were subsequently subcloned into smaller fragments which complemented the mutant strains 302 and 262 (Fig. 15). The smallest clonable fragment of pHA15 which complemented strain 302 to MMC^R was the 1.1 kb *Xho* I fragment, pHA15b.1 (Fig. 15). When sequenced, this subclone was found to include 262 bp upstream from the ATG site of an Open Reading Frame (ORF) which was highly homologous to *E. coli uvrA*. This clone also included 801 bp of the 5' terminus of an ORF. The other subclone pHA16 (2.6 kb), which restored strain 262 to MMC^R was further subcloned producing a *Sty* I 1.3 kb fragment which fully restored MMC resistance to strain 262.

Figure 15.

Map of the uvrA gene region. The cloned fragments that were employed in the transformation experiments and sequencing of the ORF (*uvrA* gene) are shown below the chromosomal map. The sizes of the cloned chromosomal fragments in pHA15, pHA15b, pHA15b.1, pHA18, pHA16 and pHA17 are indicated. A, *Acc*I; X, *Xho*I; E, *Eco*RI; S, *Sty*I.


TGA
mtcB
uvrA
mtcA
ATG
chromosome
A
X
X E
E
S
S
pHA15
5.6kb
pHA15b
3.1kb
pHA15b.1
1.1kb
pHA18
316bp
pHA16
2.6kb
pHA17
1.3kb

Earlier work (Al-Bakri et al., 1985) had suggested that pUE58 (pHA15) and pUE59 (pHA16) may encode two separate genes which could restore the defects in the two mutant strains 302 and 262, respectively, but could not restore MMC resistance in the other mutant strain. This work proves that the initial hypothesis of two genes was incorrect. In fact, each of these DNA clones restores a mutation in different regions of the same gene, *uvrA*, and these mutations may be as far as 1,000 bp apart from each other. The entire ORF is 3114 bases long corresponding to 1015 amino acids and is preceded by a likely Shine-Dalgarno ribosome binding site as well as a possible SOS box (Fig. 16).

After completely sequencing both subclones pHA15b.1 and pHA17, and adjacent sequences, it was determined that a small *EcoR* I fragment in the middle of the gene was missing. To complete the sequence, an additional *EcoR* I fragment, pHA18, was subcloned from the cosmid pUE502 which had not been previously detected because of its small size. When this clone was sequenced it corresponded perfectly to the 316 bp EcoR1 fragment in the middle of the ORF, as judged by homology to *E. coli* and *M. luteus uvrA*. This fragment did not complement either strains 302 or 262.

2. The ORF is Highly Homologous with *E. coli uvrA*.. A computer-assisted search showed that the DNA sequence homology of the *D. radiodurans* ORF with *E. coli uvrA* is 59% and the deduced amino acid sequence identity is 57%. *D. radiodurans uvrA* is actually more homologous at the nucleotide level with *M. luteus* (72%) than *E. coli uvrA*. However, at the amino acid level *D. radiodurans uvrA* is less identical with *M. luteus* (54%) versus *E. coli* (57%). The multiple alignment of the amino acid sequences from these three bacteria are shown in Fig. 17. Among all three amino acid sequences there is a 40.2% identity and 28.2% similarity, with an overall amino acid sequence homology of 68.4%.

The length of the *D. radiodurans uvrA* gene (3045 bp, 1015 a.a) is similar to that of *E. coli* (2820 bp, 940 a.a.) and *M. luteus* (3441bp, 992 a.a.) (Husain et al., 1986; Shiota

Figure 16.

Nucleotide sequence and deduced amino acid sequence of *D. radiodurans* UvrA. Base numbering is arbitrary, starting 262 bases upstream from the translation initiation site. Restriction enzyme recognition sites shown in Fig. 15 are underlined, italicized, and labeled with the name of the appropriate restriction enzyme. The *E. coli*-like possible SOS box is underlined.

Xho1
CTCGAGTATGTCGCCACTCGGC GCGGCGTGCAGGCGGTGCTGGGGCGTGCAG CTGCCTGAGGGTGATCGAGGTTGAACATCG 82

AATGATTTACCCCACGCCGCCCTCGCCGGG GCGGTTTTTCCTGCCTTTACGCAGCACATT CAGGGCCGGGCGACCTGTTAGAATATTCCG 172

TTTGAGAACGTAGGGCCAAGGCTGCCTTAC GCGCCTGCCGTAATCATGCCCTGTTGCCAA AAGGCGTGCAGGACAGCCAAAAGGAGCTTC 262

ATGCAAGACAAACTCATCGTGCGCGGCGCC CGGGAACACAACCTCAAGGACATCACCGTG GAGCTGCCCCGCGACCGCTTCGTGGTGATT 352
MetGlnAspLysLeuIleValArgGlyAla ArgGluHisAsnLeuLysAspIleThrVal GluLeuProArgAspArgPheValValIle 30

ACCGGCGTGTCGGGCAGCGGCAAGTCCACG CTGGCTTTCGACACCATCTACGCCGAGGGC CAGCGCCGCTACGTCGAGTCGCTCAGTGCC 442
ThrGlyValSerGlySerGlyLysSerThr LeuAlaPheAspThrIleTyrAlaGluGly GlnArgArgTyrValGluSerLeuSerAla 60

TACGCGAGGCAGTTCCTGGGCCTGATGGAA AAGCCCGACGTGGACAGCATCACTGGCCTC TCGCCGGCCATTTCCATCGACCAGAAGACG 532
TyrAlaArgGlnPheLeuGlyLeuMetGlu LysProAspValAspSerIleThrGlyLeu SerProAlaIleSerIleAspGlnLysThr 90

ACCAGCCACAACCCGCGCAGCACGGTGGGT ACCGTCACCGAGATTCACGACTACCTGCGC CTGCTCTACGCCCGCGTCGGTACGCCGTAT 622
ThrSerHisAsnProArgSerThrValGly ThrValThrGluIleHisAspTyrLeuArg LeuLeuTyrAlaArgValGlyThrProTyr 120

TGCCCCATCTGCGGACGCAAAATCGAAAAG CAGAGCCCCAGCGAAGTCACCGACCGCCTG CTGGCGGGCTTTCCCGACAAGCGCGCCATC 712
CysProIleCysGlyArgLysIleGluLys GlnSerProSerGluValThrAspArgLeu LeuAlaGlyPheProAspLysArgAlaIle 150

CTGCTCGCCCCGGCGGTGCGCGGACGCAAA GGCGAGTACAAGAAGCTGTTCGCTGACCTG CGACGTGAGGGCTACGCGCGGGTGCGGGTG 802
LeuLeuAlaProAlaValArgGlyArgLys GlyGluTyrLysLysLeuPheAlaAspLeu ArgArgGluGlyTyrAlaArgValArgVal 180

GACGGCACGCTCTACGAACTGGAAGAAGCC GAAAAGCTCAAGCTGGAAAAGTTCGAGAAG CACGACGTGGACATCGTGATCGACCGCCTG 892
AspGlyThrLeuTyrGluLeuGluGluAla GluLysLeuLysLeuGluLysPheGluLys HisAspValAspIleValIleAspArgLeu 210

ACTCTACGTGAGAGCGACCGCAGCCGCATC GCCGAGTCGGTGGAACTCGGCATCCGGCGC GGCGAGGGCCTGCTGCGGGTGCTGCTGCCC 982
ThrLeuArgGluSerAspArgSerArgIle AlaGluSerValGluLeuGlyIleArgArg GlyGluGlyLeuLeuArgValLeuLeuPro 240
Xho1
GACGCGGGTGAGGACGGCGGCGCGCACGAG GAGCTGTACTCCGAGAAGTTCGCCTGCCCC GAACACGGCAGCGTGCTGGAAGAACTCGAG 1072
AspAlaGlyGluAspGlyGlyAlaHisGlu GluLeuTyrSerGluLysPheAlaCysPro GluHisGlySerValLeuGluGluLeuGlu 270
EcoR1
CCGCGCTCCTTTTTCCTTCAACTCGCCCTAC GGCGCCTGCGGCGACTGCGCGGGCATCGGG GCCAAGCAGGAATTCTCGCCCGAGCGCATC 1162
ProArgSerPheSerPheAsnSerProTyr GlyAlaCysGlyAspCysAlaGlyIleGly AlaLysGlnGluPheSerProGluArgIle 300

ATCGACGAAAAACTGAGTATCGCCGGGGGC GCCATCATCCCCTGGACCAAAAAGGGCGCC GACGCGGGCATTTATTACTGGGACAAGCTC 1252
IleAspGluLysLeuSerIleAlaGlyGly AlaIleIleProtrpThrLysLysGlyAla AspAlaGlyIleTyrTyrTrpAspLysLeu 330

AAGGCGCTGGCCGAGCACCTCGACTTCGAC CTGAAAACGCCCTGGAAGGACCTGCCCGCG AAGGCGCAAAAAGCCGTGTTGCACGGCCCC 1342
LysAlaLeuAlaGluHisLeuAspPheAsp LeuLysThrProtrpLysAspLeuProAla LysAlaGlnLysAlaValLeuHisGlyPro 360

GGCGAGGCTTTTGAGGTTGTCTACCGGCGC GGCGGCAAGGAAACCATGCGCTTCATGACC GAGTTCGAGGGCGTGATCACCAACCTGGAG 1432
GlyGluAlaPheGluValValTyrArgArg GlyGlyLysGluThrMetArgPheMetThr GluPheGluGlyValIleThrAsnLeuGlu 390
EcoR1
CGGCGCTACGCAACACCGTCGGAATTCATG CGCGAGAGGCTCGAGGAACTCATGGAGCTG CGGCCCTGCCCCACCTGCGGCGGCACCCGC 1522
ArgArgTyrAlaThrProSerGluPheMet ArgGluArgLeuGluGluLeuMetGluLeu ArgProCysProThrCysGlyGlyThrArg 420

TACAAGCCCGAGATTCTGGCGGTGCGGGTG GGCGGCCTGAACATCTCGCAGACGAGCGGC ATGAGCGTGCTGGACGCCGACGCCTTTTTC 1612
TyrLysProGluIleLeuAlaValargVal GlyGlyLeuAsnIleSerGlnThrSerGly MetSerValLeuAspAlaAspAlaPhePhe 450

CAGCAGTTGCAGGAAGGCGAACTCGATCAC GCGGCCATCGAGCCCTTCCTCAAGGCCCAC ACCGGCGGCACCGCCAAGGCCACGGCCCGC 1702
GlnGlnLeuGlnGluGlyGluLeuAspHis AlaAlaIleGluProPheLeuLysAlaHis ThrGlyGlyThrAlaLysAlaHisGlyPro 480

TGCACTACGGAGTACGACCTGGGTACGTTC GGCGCGGCGGTGGCGGCCCCGATTCTGCGG GCGATTCGCACCCGGCTGAAGTTTCTGGTG 1792
LeuHisTyrGluTyrAspLeuGlyThrPhe GlyAlaAlaValAlaAlaProIleLeuArg AlaIleArgThrArgLeuLysPheLeuVal 510

GACGTGGGGCTCGACTACCTCTCGCTGGAC CGCACCGCCAACACGCTCTCGGGCGGCGAG GCGCAGCGCATCCGGCTGGCGACCCAGGTG 1882
AspValGlyLeuAspTyrLeuSerLeuAsp ArgThrAlaAsnThrLeuSerGlyGlyGlu AlaGlnArgIleArgLeuAlaThrGlnVal 540
Sty1
GGCAGCGGCCTGACCGGGGGTGCTGTACGTG CTCGACGAGCCGTCCATCGGCCTGCACCCCAAGGACAACGGGCGACTCATCGGCACGCTG 1972
GLyserGlyLeuThrGlyValLeuTyrVal LeuAspGluProSerIleGlyLeuHisPro LysAspAsnGlyArgLeuIleGlyThrLeu 570

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AAGAACCTGCGTGACCTGGGCAACTCGCTG CTGGTGGTGGAGCACGACGAGGACACCATG CTGGAGGCCGACTACCTGATCGACATGGGG 2062
LysAsnLeuArgAspLeuGlyAsnSerLeu LeuValValGluHisAspGluAspThrMet LeuGluAlaAspTyrLeuIleAspMetGly  600

CCGGGCGCGGGGGGTGCACGGCGGCGAAGTC ATCGCCTCGGGCACGCCCGAACAGGTCAAG CAGGACAAGAACAGCCTCACCGGCAAGTAC 2152
ProGlyAlaGlyValHisGlyGlyGluVal IleAlaSerGlyThrProGluGlnValLys GlnAspLysAsnSerLeuThrGlyLysTyr  630

CTGCGCGGCGAGATGAAAATCGAGGTGCCC GCCGAGCGCCGCCCCGGCAACGGCAAGTTC CTGAAGGTCTTCGGCGCGCGCCAGAACAAC 2242
LeuArgGlyGluMetLysIleGluValPro AlaGluArgArgProGlyAsnGlyLysPhe LeuLysValPheGlyAlaArgGlnAsnAsn  660

TTGCAGGACGTGGACGTGTCCATTCCGCTC GGCACCATGACGGTGGAGACTGGCCCCTCG CGCAGCGGCAAAAGCACCCTGATTCACGAC 2332
LeuGlnAspValAspValSerIleProLeu GlyThrMetThrValGluThrGlyProSer ArgSerGlyLysSerThrLeuIleHisAsp  690

ATCCTGCACGCCACGCTGGCCCGCGAACTC AACGGCGCCAAGACCACGCCGGGACTGTAC GACCGCATCGAGGGCATGGAGCAGCTGGAC 2422
IleLeuHisAlaThrLeuAlaArgGluLeu AsnGlyAlaLysThrThrProGlyLeuTyr AspArgIleGluGlyMetGluGlnLeuAsp  720

AAGGTCATCGAGATCGACCAGTCGCCCATC GGGCGCACGCCGCGCTCAAATCCGGCGACC TACACGGGCGTGTTCACCGAAATCCGTGAT 2512
LysValIleGluIleAspGlnSerProIle GlyArgThrProArgSerAsnProAlaThr TyrThrGlyValPheThrGluIleArgAsp  750

TTGTTCACCCGGACTCCCGAGGCGCGGCGG CGCGGGTATCAGGCCGGACGCTTTTCCTTC AACGTCAAGGGCGGGCGCTGCGAACACTGC 2602
LeuPheThrArgThrProGluAlaArgArg ArgGlyTyrGlnAlaGlyArgPheSerPhe AsnValLysGlyGlyArgCysGluHisCys  780

AAGGGCGACGGCGTGATGAAGATCGAGATG AACTTCCTGCCCGACATCTACGTGCCCTGC GAGGTCTGCCACGGGGCGCGCTACAATCGC 2692
LysGlyAspGlyValMetLysIleGluMet AsnPheLeuProAspIleTyrValProCys GluValCysHisGlyAlaArgTyrAsnArg  810

GAGACGCTGGAGGTCAAGTACAACCACAAG ACGATTGCCGACGTGCTCGACCTGACGGTG GAGGACGCCCACGAGTTTTTTGAGGCGATC 2782
GluThrLeuGluValLysTyrAsnHisLys ThrIleAlaAspValLeuAspLeuThrVal GluAspAlaHisGluPhePheGluAlaIle  840

CCGACCATCGAGCGCAAGATGCAACTGCTG CTCGACGTGGGCCTGGGCTACATGAAAATC GGCCAGCCCTCCACCACGCTCTCGGGCGGC 2872
ProThrIleGluArGLysMetGlnLeuLeu LeuAspValGlyLeuGlyTyrMetLysIle GlyGlnProSerThrThrLeuSerGlyGly  870

GAGGCGCAGCGCATCAAGCTGGCGACCGAG CTGAGCAAGCGGGCCACCGGGCGCACCATC TACATCCTCGACGAGCCGACCACCGGCCTG 2962
GluAlaGlnArgIleLysLeuAlaThrGlu LeuSerLysArgAlaThrGlyArgThrIle TyrIleLeuAspGluProThrThrGlyLeu  900

CACTTCGAGGACGTGCGCAAGCTGATGGAC GTGCTGCAACGCCTCGCGGAAGGTGGCAAC ACGCTGGTCATCATCGAGCACAACCTCGAC 3052
HisPheGluAspValArgLysLeuMetAsp ValLeuGlnArgLeuAlaGluGlyGlyAsn ThrLeuValIleIleGluHisAsnLeuAsp  930

GTGATGAAGTCGGCGGACTACCTCATCGAC CTGGGGCCGGAAGGCGGCGTGCGCGGCGGA ACCGTCGTGGCGGTGGGCACACCCGAAGAA 3142
ValMetLysSerAlaAspTyrLeuIleAsp LeuGlyProGluGlyGlyValArgGlyGly ThrValValAlaValGlyThrProGluGlu  960

GTCGCTGCCCACCCGACGAGCTACACCGGC GAGTACCTGCGCAAGGTGCCGGGCATCGTG GCCGCCGAGCCGCGTGCCAGGGGTGAAAAG 3232
ValAlaAlaHisProThrSerTyrThrGly GluTyrLeuArGLysValProGlyIleVal AlaAlaGluProArgAlaArgGlyGluLys  990
                Sty1
GCGGAAAAGCCCGCCAAGGCCAAAGCGCCC GCCAAGAAAAGGACCAAGAAGCAGACGGAA CTGGTCGAGGCCGACTGATAGCGGTTTGCG 3322
AlaGluLysProAlaLysAlaLysAlaPro AlaLysLysArgThrLysLysGlnThrGlu LeuValGluAlaAspTER             1015

GTTTGAAGCGCTCAATTTTGAGCGATTCAA CCCGAGCAGAGCAGAAGCAAAGAA                                    3376
```

Figure 17.

Amino acid sequence comparison of *D. radiodurans* UvrA (lower sequence) with *E. coli* UvrA (middle sequence) and *M. luteus* UvrA (upper sequence). Amino acid identities (stars) and similarities (periods) are indicated. There are five motifs; 2 ATP-binding domains, 2 Zn-binding domains and 1 helix-turn-helix. The *broken* and *solid lines* above the UvrA sequence indicate the putative ATP-binding sites and the CXXC sequences, respectively.

```
MLUTE  VPKNSSTTVSSAVEAHAGGLASGPGGARSGERDRIVVQGAREHNLKDVDVSFPRDAMVVFTGLSGSGKSSLAFDTIFAEGQRRYVESLSSYARMFLGRVD 100
ECOLI  M------------------------------DKIEVRGARTHNLKNINLVIPRDKLIVVTGLSGSGKSSLAFDTLYAEGQRRYVESLSAYARQFLSLME  69
DRAD   M------------------------------QDKLIVRGAREHNLKDITVELPRDRFVVITGVSGSGKSTLAFDTIYAEGQRRYVESLSAYARQFLGLME  70
                                      *.. *.***.****.. ...*** ..* **.******.*****..************.*** **. ..

MLUTE  KPDVDFIEGLSPAVSIDQKSTNRNPRSTVGTITEIYDYMRLLWARVGVPHCPQCGEPVSRQTPQQIVDQLEELPERTRFQVLAPVVRGRKGEFVDLFRDL 200
ECOLI  KPDVDHIEGLSPAISIEQKSTSHNPRSTVGTITEIHDYLRLLFARVGEPRCPDHDVPLAAQTVSQMVDNVLSQPEGKRLMLLAPIIKERKGEHTKTLENL 169
DRAD   KPDVDSITGLSPAISIDQKTTSHNPRSTVGTVTEIHDYLRLLYARVGTPYCPICGRKIEKQSPSEVTDRLLAGFPDKRAILLAPAVRGRKGEYKKLFADL 170
       ***** *.*****.**.**.*..********.***.**.***.**** ***   .  .. *.  ...*.. .   .*  .*** ...****  . . .*

MLUTE  STQGF--AVVDGETVQLSDPPVLKKQV--KHTIAVVVDRLAMKEGIRQRLTDSVETALKLADGLVVAEFVDVEPVAEKGKKNTAEFGGRDAEGNPRYRSF 296
ECOLI  ASQGYIRARIDGEVCDLSDPPKLELQK--KHTIEVVVDRFKVRDDLTQRLAESFETALELSGGTAV--------VADMDDPKAEEL------------LF 247
DRAD   RREGYARVRVDGTLYELEEAEKLKLEKFEKHDVDIVIDRLTLRESDRSRIAESVELGIRRGEGLLRVLLPDA-----------------GEDGGAHEELY 253
         .*.  . .**.  .*...  *. .   **....*.**. ....   *...* * ..  ..*                                    .

MLUTE  SEKLSCPNGHEQTVDEIEPRSFSFNNPFGACPECTGIGSRLQVDPDLVVANDELSLREGAVVPWSL-GKSTSDYWLRVLGGLGKEMGFSLDTPWKDLTEA 395
ECOLI  SANFACPIC-GYSMRELEPRLFSFNNPAGACPTCDGLGVQQYFDPDRVIQNPELSLAGGAIRGWD----RRNFYYFQMLKSLADHYKFDVEAPWGSLSAN 342
DRAD   SEKFACPE-HGSVLEELEPRSFSFNSPYGACGDCAGIGAKQEFSPERII-DEKLSIAGGAIIPWTKKGADAGIYYWDKLKALAEHLDFDLKTPWKDLPAK 351
       *....**   .  . *.*** ****.* *** .*.*.* .   .*. .. . .**. .**.  *.       . *.   * .*...  *....** .*..

MLUTE  ERDAVLHGKDFKVEVTFRNRFGRE----RRYTTGFEGVIPYVMRKHGETESDGARERYESFMREIPCPACHGARLNPTVLNVLVGGLSIADATRLPMREA 491
ECOLI  VHKVVLYGSG-KENIEFKYMNDRGDTSIRRHP--FEGVLHNMERRYKETESSAVREELAKFISNRPCASCEGTRLRREARHVYVENTPLPAISDMSIGHA 439
DRAD   AQKAVLHGPGEAFEVVYR----RGGKETMRFMTEFEGVITNLERRYA-TPSEFMRERLEELMELRPCPTCGGTRYKPEILAVRVGGLNISQTSGMSVLDA 446
       ...**.* .   .. ..    *.    .*    ****.  . *.. * *.  **  ....   **..* *.* ...   * *..  ... . ... .*

MLUTE  MEFFSGLR-------------------------------LTDRERQIADQVLKEILARLAFLLDVGLEYLNLERPAGTLSGGEAQRIRLATQIGSGLVG 559
ECOLI  MEFFNNLK-------------------------------LAGQRAKIAEKILKEIGDRLKFLVNVGLNYLTLSRSAETLSGGEAQRIRLASQIGAGLVG 507
DRAD   DAFFQQLQEGELDHAAIEPFLKAHTGGTAKAHGPLHYEYDLGTFGAAVAAPILRAIRTRLKFLVDVGLDYLSLDRTANTLSGGEAQRIRLATQVGSGLTG 546
       .**  *.                                 *..   .* ..*..* .** **..***.**.*.*.*.************.*.*.**.*

MLUTE  VLYVLDEPSIGLHQRDNRRLIETLLRLRDLGNTLIVVEHDEDTIAEADWIVDIGPRAGEYGGEVVHSGSLADLKANTRSVTGDYLSGRRSIAVPERRRVP 659
ECOLI  VMYVLDEPSIGLHQRDNERLLGTLIHLRDLGNTVIVVEHDEDAIRAADHVIDIGPGAGVHGGEVVAEGPLEAIMAVPESLTGQYMSGKRKIEVPKKRVPA 607
DRAD   VLYVLDEPSIGLHPKDNGRLIGTLKNLRDLGNSLLVVEHDEDTMLEADYLIDMGPGAGVHGGEVIASGTPEQVKQDKNSLTGKYLRGEMKIEVPAERRPG 646
       *.***********..** **..** .******...*******.. .** ..*.** ** .****. .*. .....   *.**.*..* ..*.**  *

MLUTE  EKGRVLTVRGAQENNLKDVSVQVPLGVLTAVTGVSGSGKSTLINEILYKVLANRLNGAKLV-PGRHRSVEGLEHLDKVVHVDQSPIGRTPRSNPATYTGV 758
ECOLI  NPEKVLKLTGARGNNLKDVTLTLPVGLFTCITGVSGSGKSTLINDTLFPIAQRQLNGATIAEPAPYRDIQGLEHFDKVIDIDQSPIGRTPRSNPATYTGV 707
DRAD   N-GKFLKVFGARQNNLQDVDVSIPLGTMTVETGPSRSGKSTLIHDILHATLARELNGAK-TTPGLYDRIEGMEQLDKVIEIDQSPIGRTPRSNPATYTGV 744
       . .. *.. **. ***.**.. .*.* .*  ** * *******...*  . .. ****. . *. .  ..*.*..***...********************

MLUTE  FDAIRKLFAETPEAKVRGYQQGRFSFNIKGGRCEACAGDGTLKIEMNFLPDVYVPCEVCHGARYNRETLEVTYKGKNIAEVLDMPIEEAADFFSAYTRIS 858
ECOLI  FTPVRELFAGVPESRARGYTPGRFSFNVRGGRCEACQGDGVIKVEMHFLPDIYVPCDQCKGKRYNRETLEIKYKGKTIHEVLDMTIEEAREFFDAVPALA 807
DRAD   FTEIRDLFTRTPEARRRGYQAGRFSFNVKGGRCEHCKGDGVMKIEMNFLPDIYVPCEVCHGARYNRETLEVKYNHKTIADVLDLTVEDAHEFFEAIPTIE 844
       *. .*.**. .**.. *** .******..***** * ***..*.**.****.****. *.* ********..*. *.* .***...*.* .**.* . ..

MLUTE  RYLDTLVDVGLGYVRLGQPATTLSGGEAQRVKLAAELQKRSNGRTIYVLDEPTTGLHFDDIRKLLHVLQSLVDKGNTVLTIEHNLDVIKSADHVIDLGPE 958
ECOLI  RKLQTLMDVGLTYIRLGQSATTLSGGEAQRVKLARELSKRGTGQTLYILDEPTTGLHFADIQQLLDVLHKLRDQGNTIVVIEHNLDVIKTADWIVDLGPE 907
UVRA   RKMQLLLDVGLGYMKIGQPSTTLSGGEAQRIKLATELSKRATGRTIYILDEPTTGLHFEDVRKLMDVLQRLAEGGNTLVIIEHNLDVMKSADYLIDLGPE 944
       * .. *.****.*...**..**********.*** ** **..*.*.*.**********.*...*..**..* . ***...*******.*.** ..*****

MLUTE  GGSGGGTIVATGTPEEVARAAESHTGRFLAELLA----------------------------------  992
ECOLI  GGSGGGEILVSGTPETVAECEASHTARFLKPML----------------------------------  940
DRAD   GGVRGGTVVAVGTPEEVAAHPTSYTGEYLRKVPGIVAAEPRARGEKAEKPAKAKAPAKKRTKKQTELVEAD  1015
       **  **.... ****.**   .*.*. .*  .
```

Figure 18.

Hydrophobicity and hydrophilicity plot of *E. coli* UvrA. A plot of the predicted secondary structure of *E. coli* UvrA is presented.

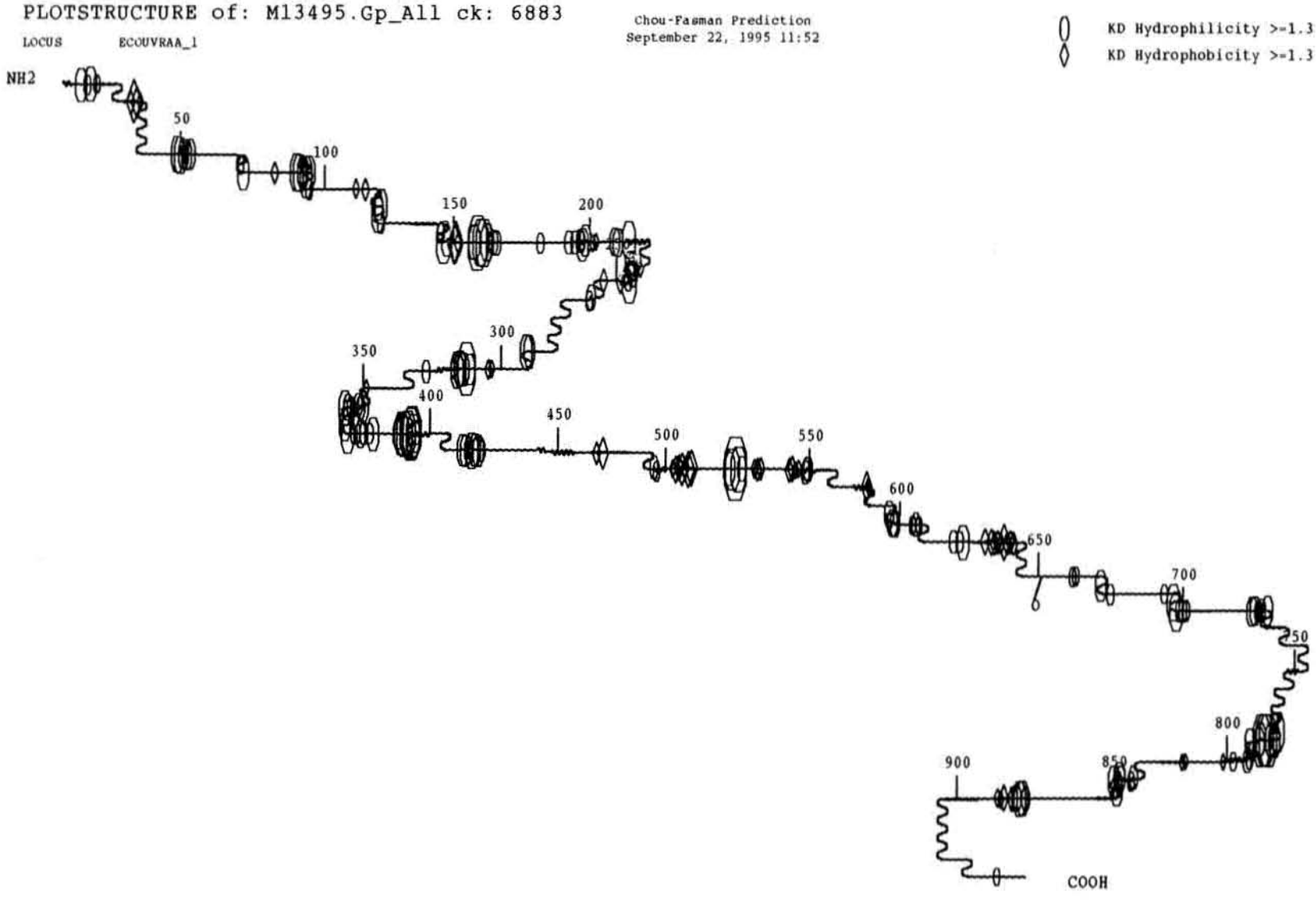
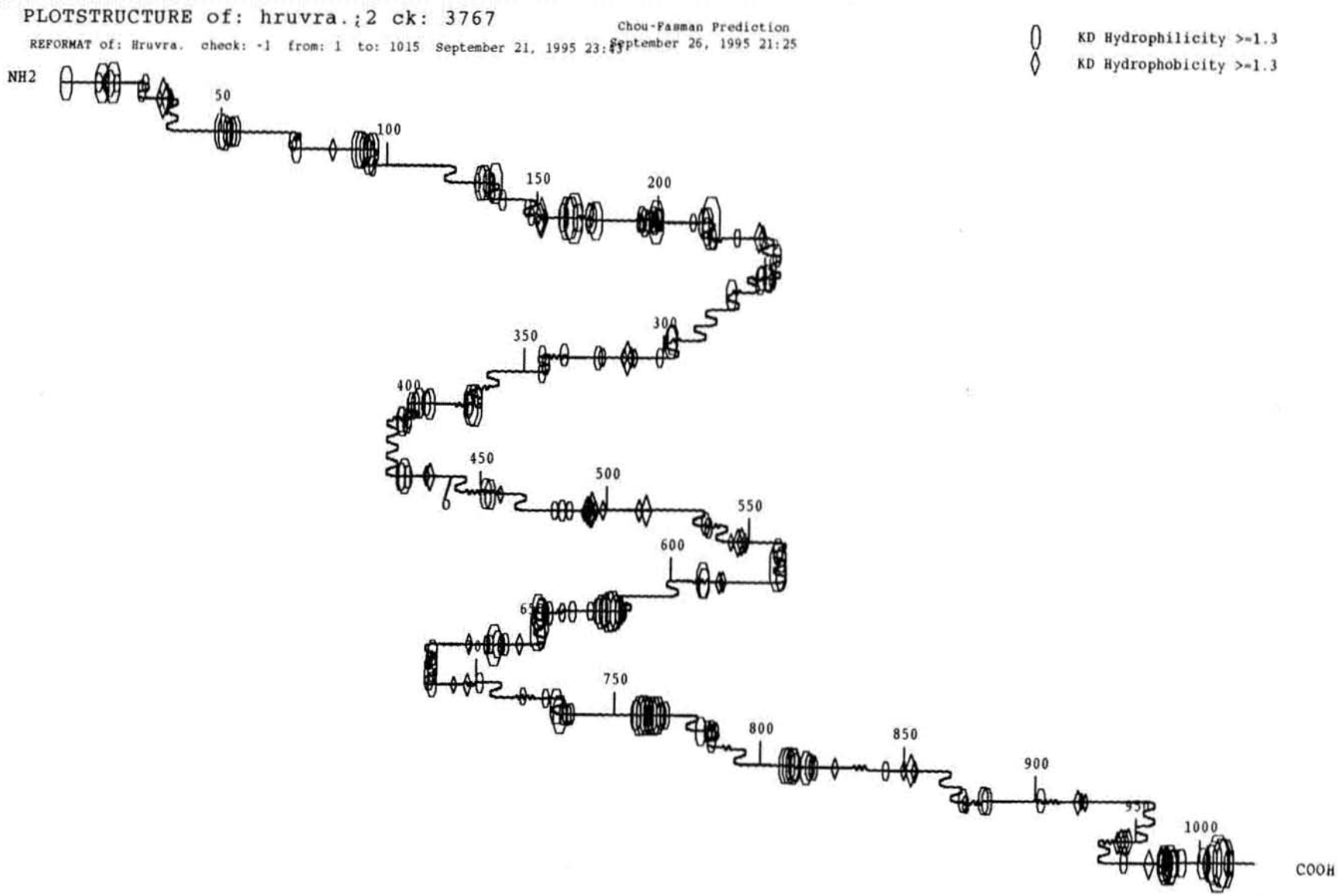

PLOTSTRUCTURE of: M13495.Gp_All ck: 6883
LOCUS ECOUVRAA_1
Chou-Fasman Prediction
September 22, 1995 11:52
KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3
NH2
50
100
150
200
300
350
400
450
500
550
600
650
700
750
800
850
900
COOH

Figure 19.

Hydrophobicity and hydrophilicity plot of *D. radiodurans* UvrA. A plot of the predicted secondary structure of *D. radiodurans* UvrA is presented.


PLOTSTRUCTURE of: hruvra.;2 ck: 3767
REFORMAT of: Hruvra. check: -1 from: 1 to: 1015 September 21, 1995 23:43
Chou-Fasman Prediction
September 26, 1995 21:25
KD Hydrophilicity >=1.3
KD Hydrophobicity >=1.3
NH2
50
100
150
200
300
350
400
450
500
550
600
750
800
850
900
1000
COOH

and Nakayama, 1989). The regions of highest amino acid homology correspond to the five motifs found in *E. coli uvrA* as well as the 5' termini and the 3' termini of the protein. The area between the first zinc-binding domain and the putative helix-turn-helix region represents the area of lowest amino acid homology. Unlike *E. coli*, *D. radiodurans* has a high G+C genomic DNA content of about 67% (Brooks and Murray, 1981), and this is reflected in the *uvrA* gene, which has a G+C content of 65%. In comparison, the G+C content of *M. luteus uvrA* is 71%. This increased use of G and C in the nucleotide sequence contributes to *M. luteus* and *D. radiodurans* having a higher homology at the nucleotide level. The secondary structures of *E. coli* UvrA and *D. radiodurans* UvrA are also shown in Figures 18, and 19. These structures are predicted on the basis of hydrophobicity and hydropholicity of amino acid residues using the algorithm of Chou-Fasman and provide limited information.

Interestingly, the *D. radiodurans uvrA* gene contains a high number of rare or infrequent codons similar to *M. luteus uvrA* that cannot be attributed solely to the high G+C content of the gene (Fig. 20). The use of rare or infrequent codons is a common occurrence in low abundance proteins. For example, the *E. coli* UvrA protein, like most DNA repair proteins in *E. coli*, is present in low copy number in the cell (about 25 molecules per cell) (Lindahl, 1982). And this protein utilizes a higher proportion of rare codons than normally seen in other genes of *E. coli* (Grosjean and Friers, 1982; Konigsberg and Godson, 1983). At the present time we do not know whether the occurrence of rare codons is the cause or result of a low abundance protein. *D. radiodurans uvrA* is also consistent with this pattern of utilizing rare codons. It is not known whether *D. radiodurans* UvrA is a low abundance protein, but that it is constitutively expressed (Evans et al., 1983). Future work with *D. radiodurans* UvrA should include studies confirming its level of expression in the cell.

3. The Five Domains of *D. radiodurans* UvrA.

The five motifs seen in *E. coli* UvrA are 2 ATP-binding domains (one at the beginning of the amino acid sequence and one near the end of the amino acid sequence); two putative Zn-binding domains and one helix-turn-helix domain (Fig. 21).

a. ATP-binding Domain. The two ATP-binding motifs found in *D. radiodurans* UvrA are Walker A-type sequences (Walker et al., 1982). These Walker A-type sequences are seen in *E.*

Figure 20.

Codon usage in *D. radiodurans* UvrA protein compared to other UvrA proteins. Rare (R) and Infrequent (I) codons are in bold print.

Codon usage in *D. radiodurans* UvrA protein compared to other UvrA protein.

Amino acid	Codon[a]	*Deinococcus radiodurans*	*Micrococcus*[b] *luteus*	*Escherichia*[c] *coli*	Amino acid	Codon	*Deinococcus radiodurans*	*Micrococcus luteus*	*Escherichia coli*
Phe	UUU	7	0	32	Thr	ACU	13	3	2
	UUC	3	2	68		ACC	4	6	55
Leu	**UUA^{I}**	**4**	**0**	**3**		**ACA^{I}**	**4**	**5**	**5**
	UUG^{I}	**5**	**0**	**7**		**ACG^{R}**	**6**	**3**	**38**
	CUU^{I}	**16**	**18**	**15**	Ala	GCU	47	39	9
	CUC^{I}	**15**	**40**	**14**		GCC	36	36	26
	CUA	21	18	1		GCA	32	35	22
	CUG	17	9	60		GCG	45	41	43
Ile	AUU	3	1	50	Tyr	UAU	4	1	52
	AUC	1	1	50		UAC	5	3	48
	AUA^{R}	**1**	**0**	**0**	His	CAU	51	30	48
Met	AUG	0	0	0		CAC	36	44	52
Val	GUU	15	18	19	Gln	**CAA^{R}**	**54**	**47**	**26**
	GUC	10	24	18		CAG	15	6	74
	GUA	10	7	9	Asn	**AAU^{R}**	**5**	**0**	**33**
	GUG	3	15	54		AAC	5	0	67
Ser	UCU	4	10	15	Lys	AAA	11	0	65
	UCC	6	9	26		**AAG^{I}**	**6**	**0**	**35**
	UCA^{I}	**7**	**4**	**13**	Asp	GAU	20	23	50
	UCG^{R}	**3**	**11**	**19**		GAC	24	19	50
	AGU^{I}	**3**	**2**	**6**	Glu	GAA	24	24	62
	AGC	5	2	21		**GAG^{I}**	**12**	**13**	**38**
Pro	**CCU^{R}**	**40**	**58**	**11**	Cys	UGU	1	6	14
	CCC^{R}	**15**	**34**	**2**		UGC	7	8	86
	CCA	15	28	17	Trp	UGG	0	8	0
Gly	GGU	31	35	29	Arg	CGU	34	71	44
	GGC	39	40	52		CGC	43	55	45
	GGA^{I}	**45**	**75**	**4**		**CGA^{I}**	**92**	**71**	**5**
	GGG^{I}	**38**	**42**	**15**		**CGG^{I}**	**60**	**79**	**6**
						AGA^{I}	**5**	**1**	**0**
						AGG^{R}	**6**	**7**	**0**

[a]R= rare, I= infrequent codons.
bFrom Shiota and Nakayama, 1989
[c]From Husain et al., 1986

Figure 21.

The five major motifs of the *D. radiodurans* UvrA protein. Amino acid sequences positions are indicated above and below the various domains.

***D. radiodurans* UVRA**

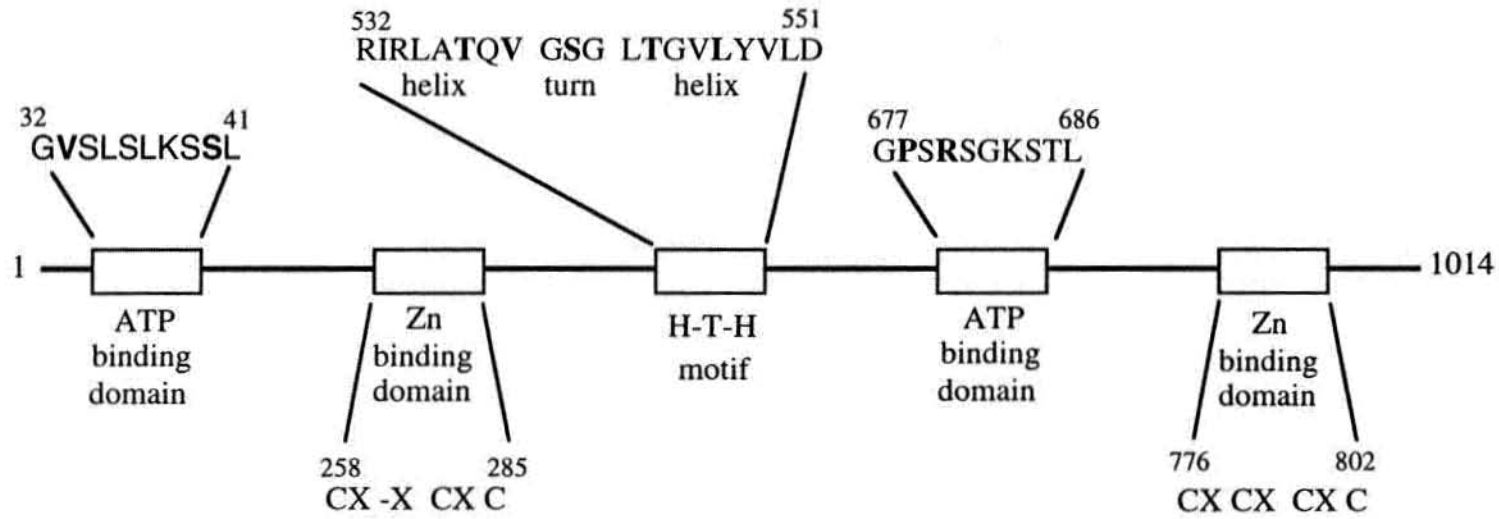


Figure 22.

ATP-binding motifs in the UvrA protein. The consensus sequence of Walker et al. (1982) is in *solid boxes*.

Fig. 22. ATP-binding motifs in the UvrA protein.

1st ATP-binding motif

M. luteus	62-76	:**G** LSGS **GKS** SLAFDT **I**
E. coli	24-45	:**G** LSGS **GKS** SLAFDT **L**
D. radiodurans	25-46	:**G** VSGS **GKS** TLAFDT **I**

2nd ATP-binding motif

M. luteus	692-706	:**G** VSGS **GKS** TLINEI **L**
E. coli	740-754	:**G** VSGS **GKS** TLINDT **L**
D. radiodurans	678-692	:**G** PSRS **GKS** TLIHDI **L**

Walker A-type sequence: **G**–X_4–**GKT**–X_6–**I**

coli as well as *M. luteus* (Fig. 22). Walker et al., 1982 found that the sequence G-X_4-GKT-X_6-I was present in a number of ATPases and suggested that this sequence might constitute part of a nucleotide binding site. In addition to the A-type sequence some ATPases contain a B-type sequence consisting of R-X-G-X_3-(hydrophobic)$_4$-D. Some ATPases have both type sequences and others have one or the other. As mentioned, *D. radiodurans*, *E. coli* and *M. luteus* UvrAs have only the A-type sequence.

b. Zn-binding Domains. In *E. coli* UvrA, the two cysteine-rich regions contain the sequence Cys-X_2-Cys-X_{19-20}-Cys-X_2-Cys. Due to the large intercysteinic region and the lack of specific conserved residues (X_2 regions) they do not belong to one of the main classes of zinc-binding domains (Berg, 1990). The N-terminal part of the first zinc-binding domain of *E. coli* uvrA is not conserved in the homologous *Micrococcus luteus* (Shiota and Nakayama, 1989) or *D. radiodurans* UvrA (Fig. 23). However, in *M. luteus* and *D. radiodurans* UvrA the structure of the zinc-binding domain might be conserved as a cysteine and a histidine. Large intercysteinic regions are also found in other enzymes such as aspartate transcarbamoylase regulatory subunit (Honzatko et al., 1982) and poly(ADP-ribose)polymerase (Uchida et al, 1987).

Recent in vitro work in *E. coli* has determined that when the first zinc binding domain is removed by mutagenesis, ATPase activity, helicase activity, DNA binding, incision of damaged DNA and DNA repair synthesis are not affected in the normal functioning of UvrA (de Ruijter et al., 1993). Therefore, it has been determined that the first zinc-binding domain of *E. coli* UvrA is not essential for DNA excision repair. It is likely that the same is true for *D. radiodurans* and M. luteus UvrA as well.

c. Helix-turn-helix Domain. A putative helix-turn-helix domain is located from amino acid position 532 to 552 (Fig. 17). This particular sequence in *D. radiodurans* UvrA is fairly identical to the helix-turn-helix region in *E. coli* and *M. luteus*. In *E. coli* this sequence does not follow the formula for a classical helix-turn-helix region and its actual function as a DNA binding domain is questionable (Brennan and Matthews, 1989; Dodd and Egan, 1990;

Figure 23.

Zn-binding domains in the UvrA proteins of several species. Note that *M. luteus* includes a histidine at the fifth position instead of the fourth position as seen in *E. coli*. *D. radiodurans* also includes a histidine at the fourth position in the first Zn-binding domain which may function in the place of a cysteine.

Fig. 23. Zn-binding domains in the UvrA protein.

1st Zn-binding domain

M. luteus	302-331	:**C**PNG**H**	X20	**C**PE**C**
E. coli	253-281	:**C**PI**C**	X20	**C**PT**C**
D. radiodurans	259-286	:**C**PE**H**	X20	**C**GD**C**

2nd Zn-binding domain

M. luteus	791-817	:**C**EA**C**	X19	**C**EV**C**
E. coli	739-766	:**C**EA**C**	X19	**C**DQ**C**
D. radiodurans	776-803	:**C**EH**C**	X19	**C**EV**C**

Claassen et al., 1991). In fact, helix-turn-helix domains in general bind to DNA at specific sequences and it seems unlikely that sequence-specific DNA interactions play a role in the binding of *E. coli* UvrA to a damaged site in the DNA, since UvrA recognizes a great spectrum of DNA damage (Pabo and Sauer, 1984).

To date, it appears that the most likely candidates for DNA binding interactions more plausibly appear to be the ATP binding domains. In fact, determining how UvrA is capable of recognizing not only helix distorting but also non-helix distorting lesions is a very active area of research at the present time.

d. SOS Operator Region. *D. radiodurans uvrA* possesses an SOS operator region within 100 bp of the translation start site of the gene (Fig. 16). The operator region in *E. coli* uvrA is shown in Fig. 24. It is evident that *D. radiodurans* UvrA possess all but one of the conserved nucleotides in the two consensus boxes. Panel B in Fig. 24 shows mutations in the consensus box which result in constitutive expression of the respective genes listed (Friedberg et al., 1995). At present there are no known operator constitutive uvrA mutants in *E. coli*. Interestingly, *D. radiodurans uvrA* is constitutively expressed (similar to the other uvrA genes identified to date) and the reason for this constitutive expression may be the single-base alteration in the putative operator region (Evans and Moseley, 1985). Future studies should address the role of the SOS operator, if any, in the expression of UvrA in *D. radiodurans*.

Additionally, the LexA repressor (which normally binds to this operator region) has yet to be identified in *D. radiodurans*. Are regulation of DNA repair proteins in *D. radiodurans* NER pathways similar to that seen in *E. coli* ? We currently understand that *D. radiodurans* UvrA is not induced (Fig. 27), but this does not suggest that a regulatory mechanism does not exist for the expression of the other DNA repair genes which might be present in this pathway e.g. *uvrB, uvrC, lexA, recA*.

Figure 24.

Presumptive SOS box from *D. radiodurans uvrA* gene compared to other SOS boxes. Panel A. SOS nucleotide sequences from various species as marked. Conserved sequences are in *bold lettering*. **Panel B.** Single base operator constitutive mutants observed from various sequences as listed. Nucleotides in *bold lettering* are positions where mutations have been made resulting in operator constitutive phenotypes. Where there is more than one bold letter in a sequence this indicates that multiple independent mutations (each bold letter alone) produces the operator-constitutive defect.

Fig. 24. Presumptive SOS box from *D. radiodurans* uvrA gene.

A.

D. radiodurans uvrA	AC **CTGT** TAGAATATT **CCG** TT
E. coli uvrA consensus sequence	ta **CTGT** atata-a-a **CAG** ta

B.

O^c Mutations	5'	TA CTGT ATATATAT ACAG TA 3'
O^c *mucAB*		TA **T**TGT ATATATAT ACAG TA
O^c *recA*		TA C**C**GC ATATATA**A** **GTG**G TA
O^c *lexA*		TA **TTTT** ATATATAT **ATGA** TA
O^c *umuDC*		TA CT**A**T ATATATAT **ATAA** TA

B. Expression of *E. coli* uvrA Homolog in *D. radiodurans* mutant Strains 302 and 262.

Since the amino acid homology between *E. coli* and *D. radiodurans uvrA* seemed to be high with all five motifs reasonably conserved we wanted to determine whether there was any functional differences between the two proteins. To answer this question we decided to transform the *E. coli uvrA* gene into *D. radiodurans* strains 302 and 262. To meet this goal the *E. coli uvrA* complete coding sequence including the promoter region cloned in pSST10 was obtained from Lawrence Grossman, John Hopkins University (Baltimore, Maryland). The gene was excised from pSST10 (Fig. 25) by *BamH* I and *Hind* III digest, filled-in and then ligated into the *Dra* I- *Bgl* II site of the deinococcal integration vector pS11 (Smith et al., 1988, Gutman et al., 1991; Gutman et al., 1994b). As previously mentioned pS11 is an originless vector which when transformed into *D. radiodurans* recombines with the genome via duplication insertion. Under treatment with kanamycin the inserted sequence is amplified up to 50 copies per chromosome along with the kanamycin resistance gene.

To confirm that the *E. coli uvrA* gene (pS11::*uvrA*) had integrated into the genomes of the MMC mutant strains 302 and 262 restriction digests of genomic DNA from kanamycin resistant colonies was performed with *Xba* I and *Xho* I, and electrophoresed through an agarose gel (0.8%). Fig. 26 is a southern blot showing the presence of pS11::*uvrA* in the *D. radiodurans* genome. The probe used was an *E. coli uvrA* (*Xba* I-*Xho* I) fragment which had been previously labeled by nick-translation with dCTP-^{32}P. As shown in Fig. 26, the *E. coli* uvrA gene had successfully integrated into the genomes of 302 and 262. *D. radiodurans* wild-type genomic DNA as well as mutant strains 302 and 262 genomic DNA were run as negative controls. The restriction digested (*Xho* I, *Xba* I) plasmids pS11 and pS11::*uvrA* were included as size markers as well as positive controls.

Figure. 25.

A. Construction of pS11::*uvrA*. A. 4.0-kb *Hind*III-*BamH*I segment that contains the *E. coli uvrA* gene was inserted in pS11. The fragment was first cut with *Hind*III, filled-in, then cut with *BamH*I. The vector, pS11, was cut with *Dra*I and *Bgl* II. The *uvrA* fragment was directionally cloned into the vector. The *Bam*HI site of the fragment was compatible with the *Bgl* II site of pS11. The *Dra*I site of the vector was destroyed by cloning of the filled-in *Hind*III terminus into this site.

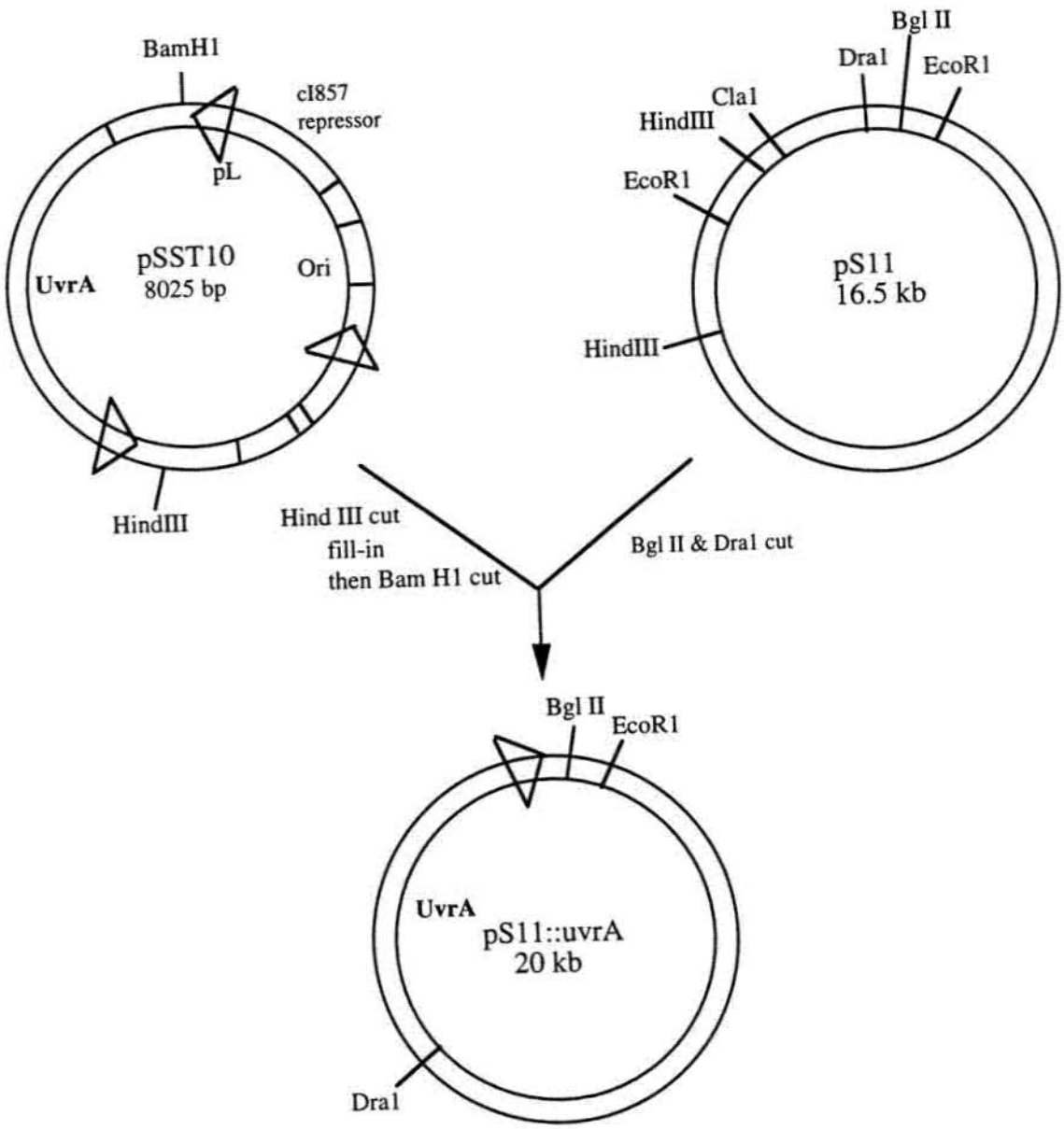
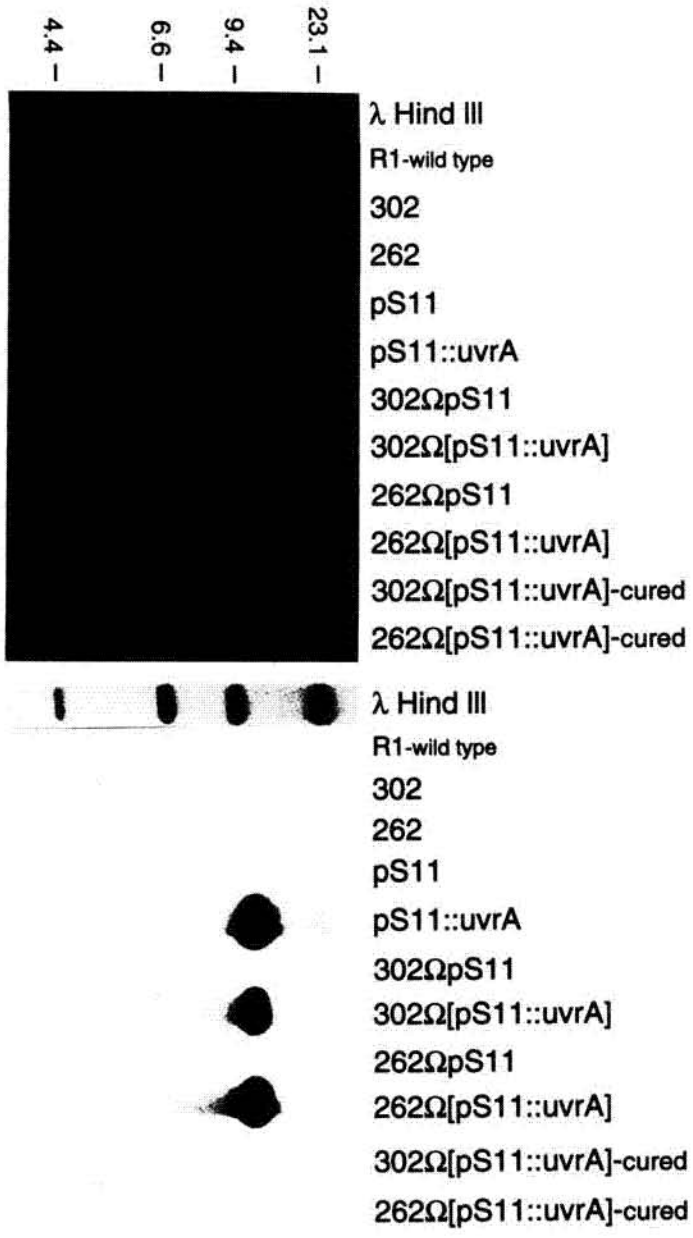

BamH1
cI857
repressor
pL
pSST10
8025 bp
UvrA
Ori
HindIII
Bgl II
Dra1
EcoR1
Cla1
HindIII
EcoR1
pS11
16.5 kb
HindIII
Hind III cut
fill-in
then Bam H1 cut
Bgl II & Dra1 cut
Bgl II
EcoR1
UvrA
pS11::uvrA
20 kb
Dra1

Figure 26.

Amplification of *E. coli* uvrA gene in various *D. radiodurans* strains. **Panel A**. Agarose gel (0.5%) showing *Deinococcus radiodurans* chromosomal digest using *Xho* I and *Xba* I restriction enzymes. **Panel B.** Southern blot of the agarose gel in Panel A. The *uvrA Xho* I to *Xba* I fragment was labeled with ^{32}P-dCTP and was used as a probe in Panel B to detect the presence of *uvrA* in strains 302 and 262.

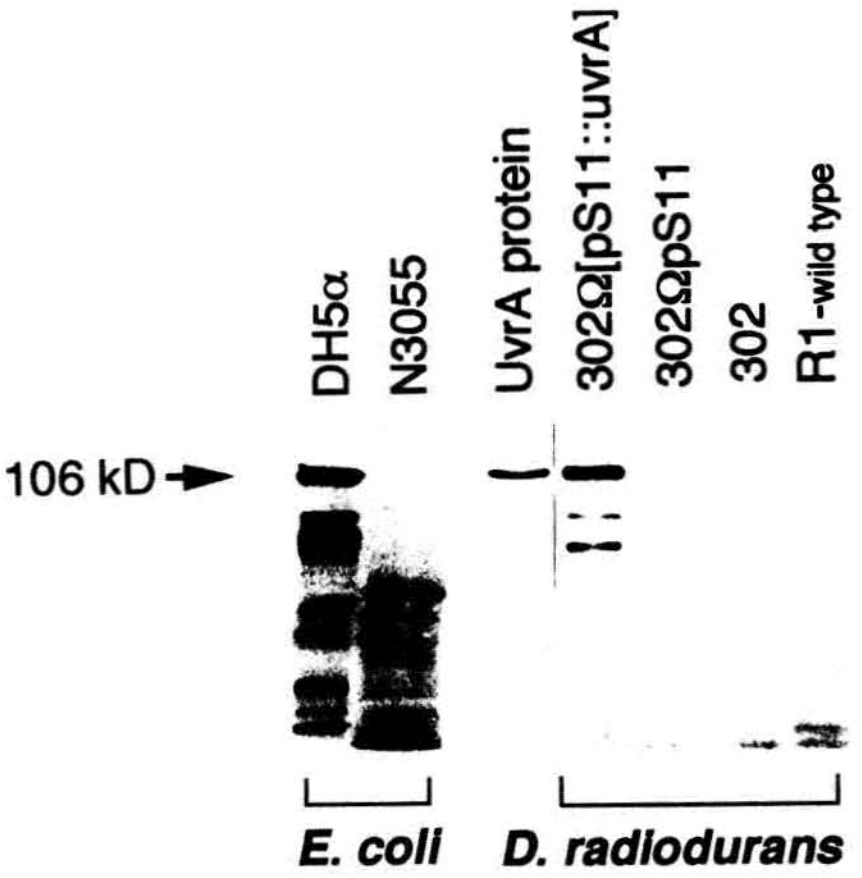

4.4 –
6.6 –
9.4 –
23.1 –
λ Hind III
R1-wild type
302
262
pS11
pS11::uvrA
302ΩpS11
302Ω[pS11::uvrA]
262ΩpS11
262Ω[pS11::uvrA]
302Ω[pS11::uvrA]-cured
262Ω[pS11::uvrA]-cured
λ Hind III
R1-wild type
302
262
pS11
pS11::uvrA
302ΩpS11
302Ω[pS11::uvrA]
262ΩpS11
262Ω[pS11::uvrA]
302Ω[pS11::uvrA]-cured
262Ω[pS11::uvrA]-cured

Once *E. coli uvrA* had successfully integrated into the genomes of the two mutant strains 302 and 262 we wanted to confirm expression of the UvrA protein. To meet this end we tested several anti-UvrA antibodies for their ability to bind to *E. coli* UvrA. The antibodies, two polyclonal and one monoclonal anti-UvrA antibody were obtained from Oleg Kovalsky and Lawrence Grossman, Johns Hopkins University, (Baltimore, Maryland). After multiple manipulations of wash conditions and reagent concentrations we were unable to obtain western blots which had reasonably low levels of nonspecific binding of the polyclonal anti-uvrA antibodies. However, the monoclonal antibody A2A3 (raised to amino acid sequence 630-940) bound very specifically to the *E. coli* UvrA protein and gave little background signal. This monoclonal was used in the subsequent experiment.

Three different methods for liberating intracellular proteins from the *D. radiodurans* cells were also tested. Initially, the cells were boiled in SDS loading buffer and loaded onto SDS-page gels. The resulting westerns displayed a weak UvrA signal with a great amount of degradation products. Secondly, the intracellular proteins were liberated from the *D. radiodurans* cells by sonication, but this treatment was too harsh resulting in a greater number of degradation products than by lysis with SDS-page buffer. Finally, intracellular proteins were extracted from the cells by the French press method (refer to Methods section). This method proved the most successful and resulted in very clean Western blots with low background signal. By using the French pressure cell method a greater concentration of protein (100ug/well) was subsequently loaded onto the SDS-page gels resulting in a very strong UvrA signal.

Figure 27.

Expression of *E. coli* UvrA protein in *E. coli* and *D. radiodurans* as assessed by Western blot.


106 kD
DH5α
N3055
UvrA protein
302Ω[pS11::uvrA]
302ΩpS11
302
R1-wild type
E. coli
D. radiodurans

To confirm that *E. coli* uvrA was expressed in *D. radiodurans* mutant strains 302 and 262 the following experiment was conducted. *E. coli* DH5α and *D. radiodurans* R1 (wild-type) and other strains were grown in liquid culture for 2 days. The cultures were divided in half and one group was incubated with MTC (1ug/ml for *E. coli* and 5 ug/ml for *D. radiodurans*) for 2 hours. At the end of this time the treated cells were washed, all cells were pelleted and then French pressed (900 psi), centrifuged at 100,000 rpm for 1 hour. Protein concentration was adjusted and about 100ug protein was loaded per well.

As shown in Fig. 27., *E. coli* UvrA is not increased with MTC treatment. *E. coli* DH5α cells are *recA*, and therefore the DNA repair genes such as *uvrA*, and *uvrB* are not induced upon treatment with MTC. What is shown in the DH5α cells is the basal constitutive level of expression of *E. coli* UvrA. And as stated earlier, about 25 molecules of UvrA per cell exist in the uninduced state (Husain et al., 1986). After induction this level is increased 5-fold (Husain et al., 1986). In *D. radiodurans* 302Ω[pS11::*uvrA*] one can see the expression of UvrA clearly. We did not expect to see the induction of UvrA in 302Ω[pS11::uvrA] because expression of this gene is under the control of the promoter in pS11 and not under the control of an "SOS" regulon. As can be seen in this figure, the monoclonal anti-UvrA antibody A2A3 does not detect *D. radiodurans* UvrA in R1. Unfortunately, neither the polyclonal nor monoclonal antibodies to *E. coli* uvrA cross-reacted with *D. radiodurans* uvrA.

Previous work in this field (Evans and Moseley, 1983) had indicated that *D. radiodurans uvrA* is not induced by DNA damage. This finding has not been supported with a Western blot, demonstrating lack of UvrA increase. Future work on *D. radiodurans uvrA* should focus on understanding expression of this gene and the other DNA repair genes in this pathway, since lack of induction appears to be the only remarkable difference between the *E. coli uvrA* and *D. radiodurans uvrA*. In summary, *E. coli* UvrA protein is successfully expressed in *D. radiodurans* strain 302.

C. Treatment with Various DNA Damaging Agents.

D. radiodurans 302 is mutant in a single gene, *mtcA* (*uvrA*), which plays a major role in the repair of MTC-induced damage, but not UV-induced unless the strain is also lacking UV endonuclease-β i.e. *uvsC*, *uvsD*, or *uvsE* (see Introduction) (Moseley and Evans, 1983; Evans and Moseley, 1983). This gene must also be active in the repair of various lethal and mutagenic lesions to which 302 is hypersensitive, either because it encodes a single enzyme capable of recognizing some common feature of differently modified nucleotides or because it controls or interacts with several enzymes initiating repair of a wide range of defects.

Strain 302 is sensitive to a wide range of mutagens which cause major helical distortions as well as those which do not cause helical distortions but attach to exocyclic N groups. For example, compounds that cause major helical distortions in *D. radiodurans* include the following, MTC, DCMTC, BrMBA and AAAF. AAAF and BrMBA cause arylalkylation of exocyclic amino-groups of purines and cytosines which for both mutagens leads to denaturation of the double-helix (Grunburger et al, 1974; Cerutti, 1975), resulting in a nonpairing base. *E. coli* mutagenesis by these compounds is sparing and depends entirely on error-prone repair translesional semiconservative DNA synthesis repair. Strains unable to remove the modified bases are sensitive to the lethal effects of these agents and are not mutated (Kondo et al., 1970; Murayama and Otsuji, 1973; Tarmy et al., 1973). The absolute immutability of *D. radiodurans wild-type* and (302) by these compounds (MTC, DCMTC, BrMBA, and AAAF) confirms the viewpoint that *D. radiodurans* lacks an *E. coli* type error-prone repair pathway. An error-prone pathway would be essential for inducing mutation by agents producing non-pairing, as opposed to mispairing nucleotides.

In addition to the major helical distortions caused by the above mentioned mutagens, some of these compounds also cause non-helical distorting lesions which are not repaired in

strain 302 but are repaired in the wild-type. For example, in addition to the AAAF adduct at the C^8 position of guanine, AAAF is also substituted at the exocyclic N^2 position of guanine, an addition which creates little or no distortion of the DNA helix (Yamasaki et al., 1977; Beland, 1978).

In this dissertation, we wanted to examine several mutagens which cause helical distorting as well as non-helical distorting lesions in strains 302Ω[pS11::*uvrA*]. Questions to be answered were: Can *E. coli uvrA* functionally replace *D. radiodurans uvrA*? If so, then this implies that *D. radiodurans* UvrA does not possess special repair capabilities, and enhanced efficiency of NER repair must be attributed to other protein(s) or pathways.

To address this question, *D. radiodurans* 302, 302Ω[pS11::*uvrA*] and *wild-type* cells were treated with various DNA damaging agents and cell survival was examined for each strain.

1. MTC Treatment:

In Fig. 28 *D. radiodurans* strains were treated with 5ug/ml MTC as indicated in the Materials and Methods section. As shown, strain 302ΩpS11::*uvrA* was as resistant to MMC$^-$ induced damage as the wild-type cells. This finding was also seen in strain 262ΩpS11::uvrA. This experiment was repeated multiple times with the same results. From this experiment, one can draw the conclusion that *E. coli uvrA* can functionally replace *D. radiodurans uvrA*. This also indicates that *D. radiodurans uvrA* is not special in it recognition of MTC-inflicted DNA damage and that there must be some other component of the UvrABC pathway that enables *D. radiodurans* to repair such large quantities of DNA damage. Normally, *E. coli* can repair less than 1/10th the dose of MTC-inflicted damage as *D. radiodurans*. But when *E. coli* UvrA is placed in the *D. radiodurans* system, *D. radiodurans* can utilize this enzyme to presumably recognize and excise 10 times the number of MTC-inflicted DNA damage sites than it could repair in the *E. coli* system.

2. 4NQO Treatment:

In Fig. 29, the cell cultures are treated with another "bulky" (helix distorting) adduct, 4NQO. This chemical attaches to the C^8, and N^2-guanine positions (see Fig. 6), creating major helical distortion. As seen with MTC, 302ΩpS11::*uvrA* is just as resistant as wild-type to these sorts of lesions. Consistent with the MTC results this means that *D. radiodurans* UvrABC exonuclease system can utilize this *E. coli* UvrA to recognize DNA damage that will subsequently be excised.

Figure 28.

Cell Survival following MTC Treatment.

Cell Survival following MTC Treatment

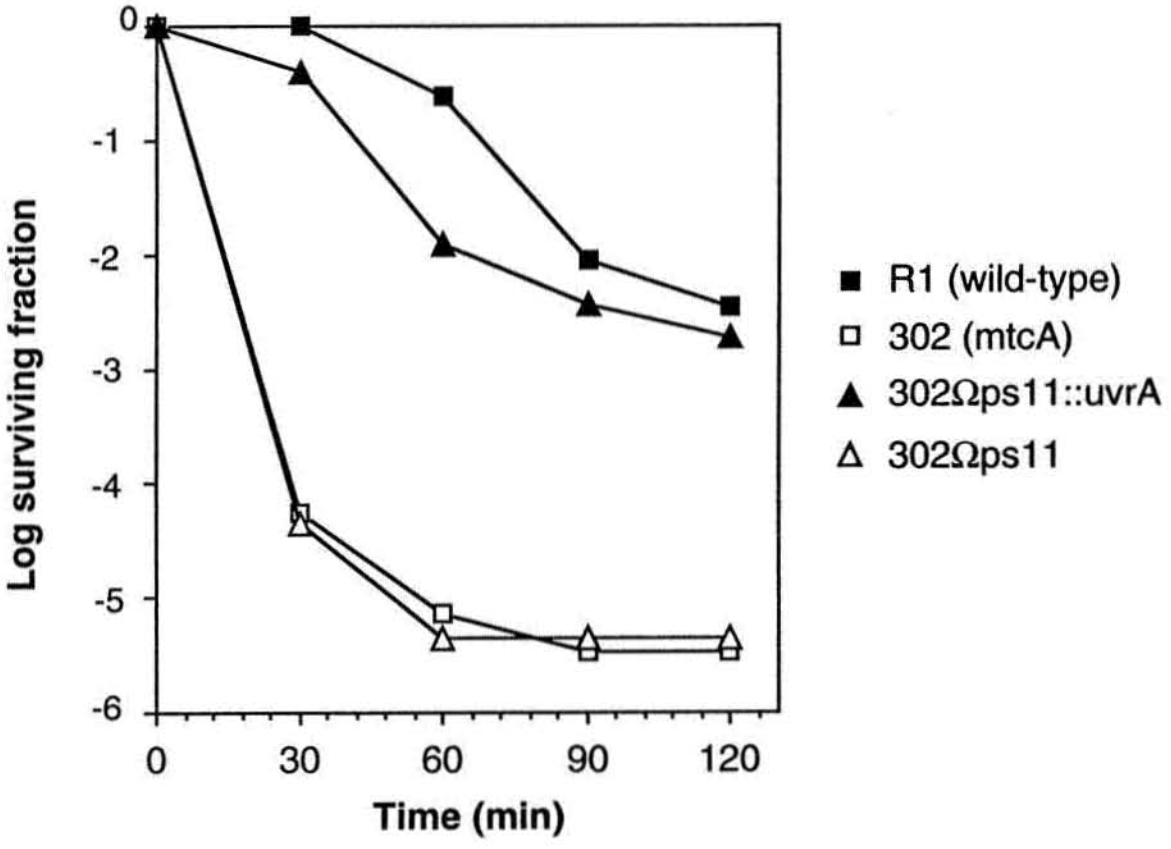
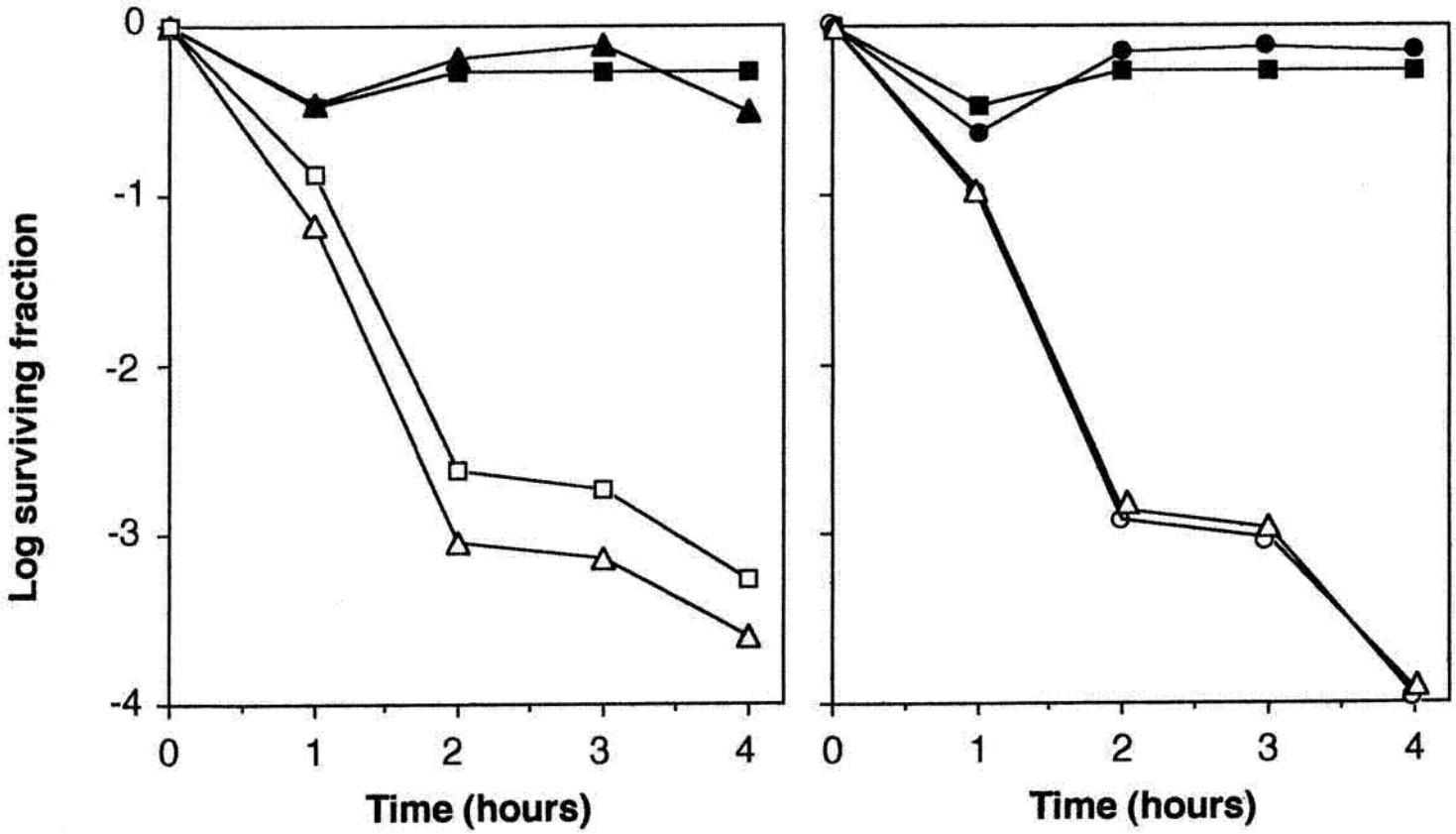


Figure 29.

Cell Survival following 4NQO Treatment.

Cell Survival following 4NQO Treatment



3. Psoralen + UV Treatment:

Psoralen causes chiefly monoadducts to the helix, but when near UV light is administered these monoadducts are converted to interstrand cross-links (Friedberg et al., 1995). These interstrand cross-links are then removed via the UvrABC pathway in *E. coli.* As shown in Fig. 30 strains 302ΩpS11::*uvrA* and 262ΩpS11::*uvrA* are again as resistant as wild-type to psoralen cross-links.

4. UV Treatment:

In Fig. 31, *D. radiodurans* cells were exposed to various doses of UV irradiation (254nm) as described in the Materials and Methods. This data supports previous work by Moseley and Evans (1983) and Evans and Moseley (1983) that demonstrated that the uvrA- strain 302 is not sensitive to UV radiation. As previously mentioned, two excision repair pathways are believed to exist in *D. radiodurans.* The pathway remaining active in 302 is the UV endonuclease-β pathway (UvsCDE) which can repair UV-induced DNA damage as well as wild-type strain R1. Not until both pathways are inactivated, one mutant in *mtcA* (*uvrA*) and the other mutant in *uvsCDE* does one see a UV sensitive phenotype (Evans and Moseley, 1983). In the case where *E. coli uvrA* is expressed in *D. radiodurans* strain 302 (302Ω[pS11::*uvrA*]) one sees no sensitivity to UV radiation, as anticipated (not shown).

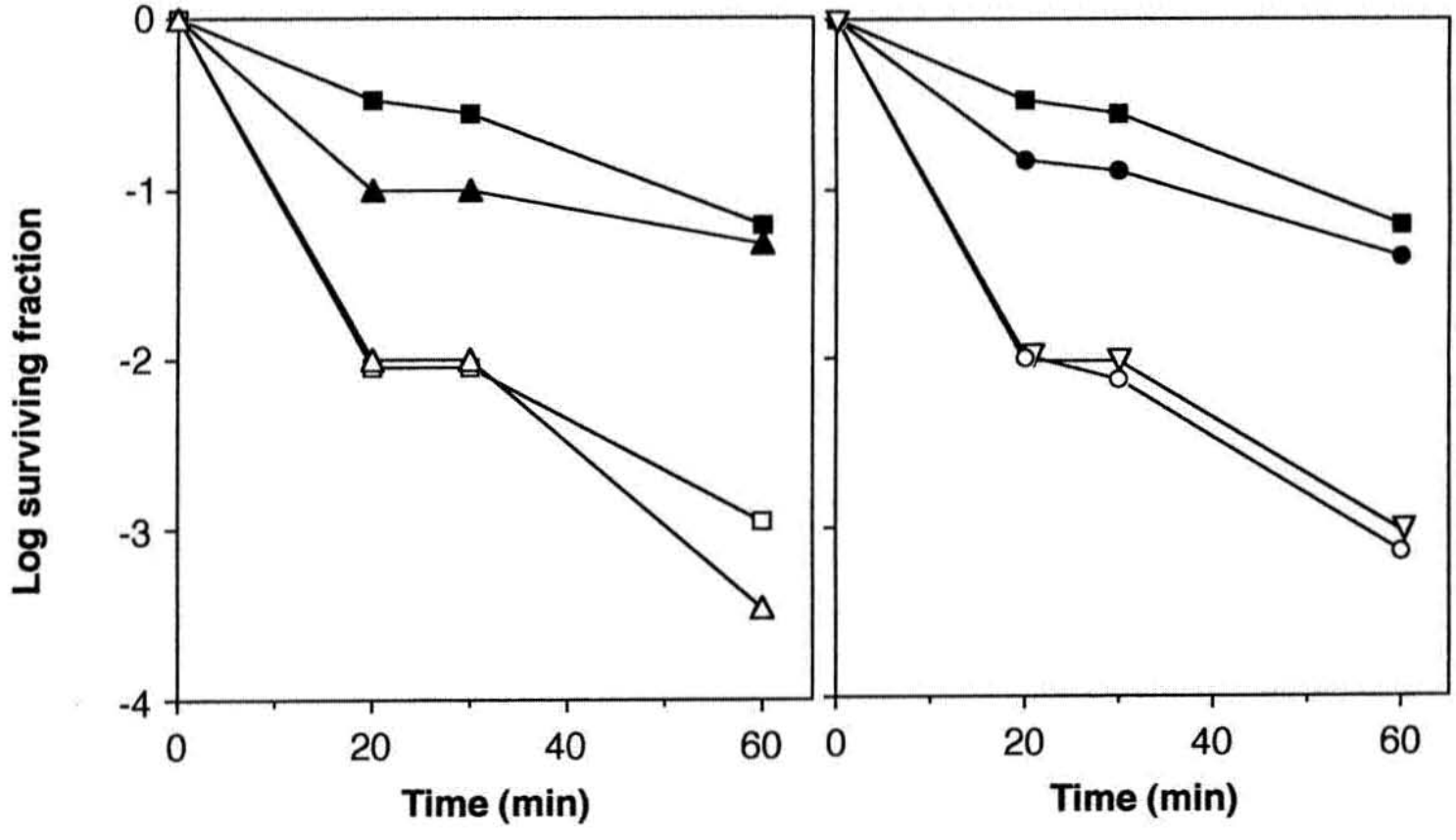
5. Ionizing Radiation Treatment:

In Fig. 32, *D. radiodurans* cells were exposed to various doses of ionizing radiation treatments as described in Materials and Methods. As demonstrated in previous studies (Evans and Moseley, 1983, Moseley and Evans 1983) *uvrA* strain 302 and 262 are not sensitive to the effects of ionizing radiation. This is because ionizing radiation does not cause helix distorting lesions and the damage caused by ionizing radiation is repaired via other pathways (*e.g.*, thymine glycol glycosylase initiated base excision repair).

Figure 30.

Cell Survival following Psoralen + near UV Radiation Treatment (320 nm)

Cell Survival following Psoralen + UV (120 Joules)



- ■ R1 (wild-type)
- □ 302 (mtcA)
- ▲ 302Ωps11::uvrA
- △ 302Ωps11

- ■ R1 (wild-type)
- ○ 262 (mtcB)
- ● 262Ωps11::uvrA
- ▽ 262Ωps11

Figure 31.

Cell Survival following far UV Radiation Treatment (254nm).

Cell Survival following UV treatment

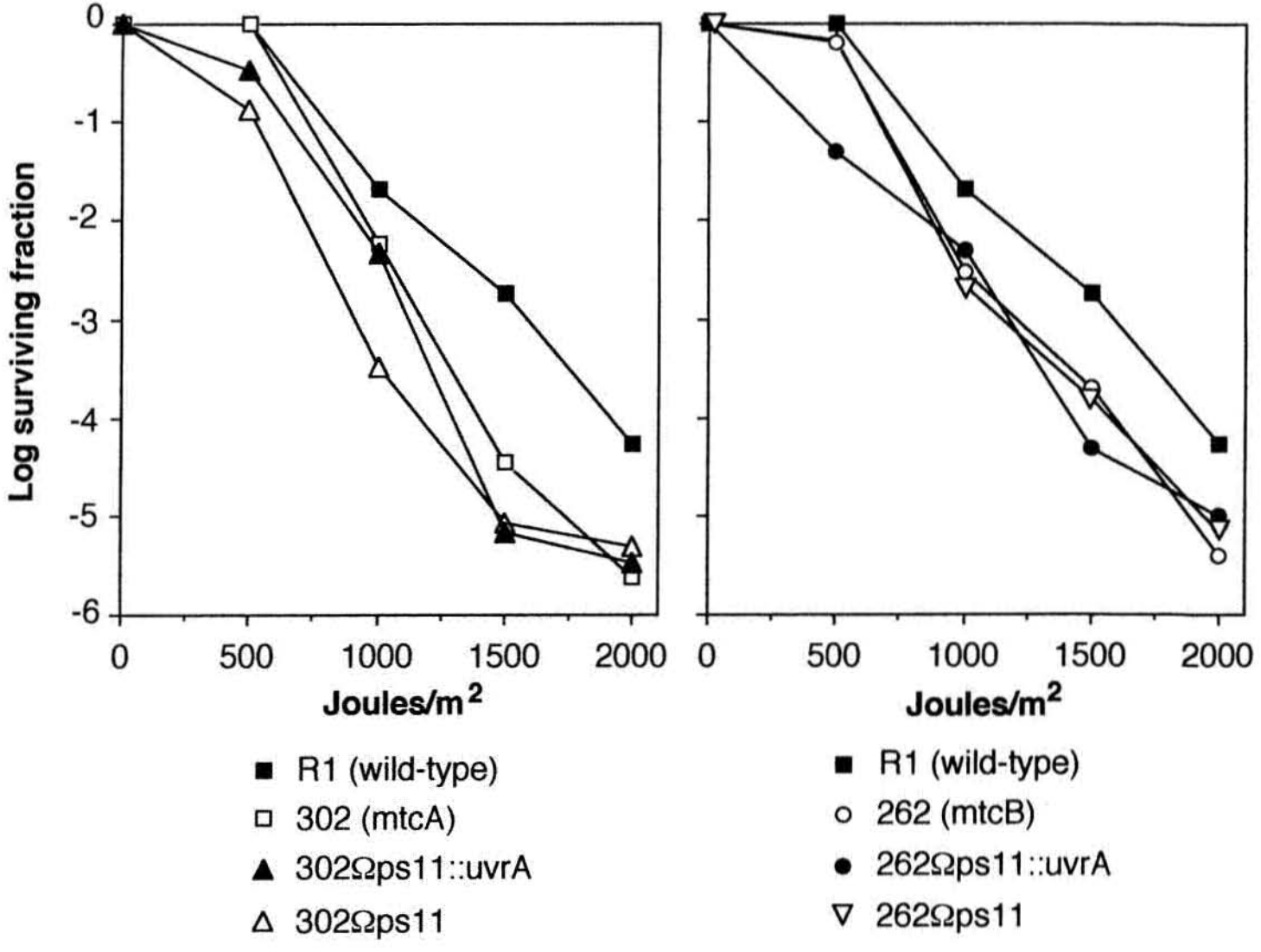
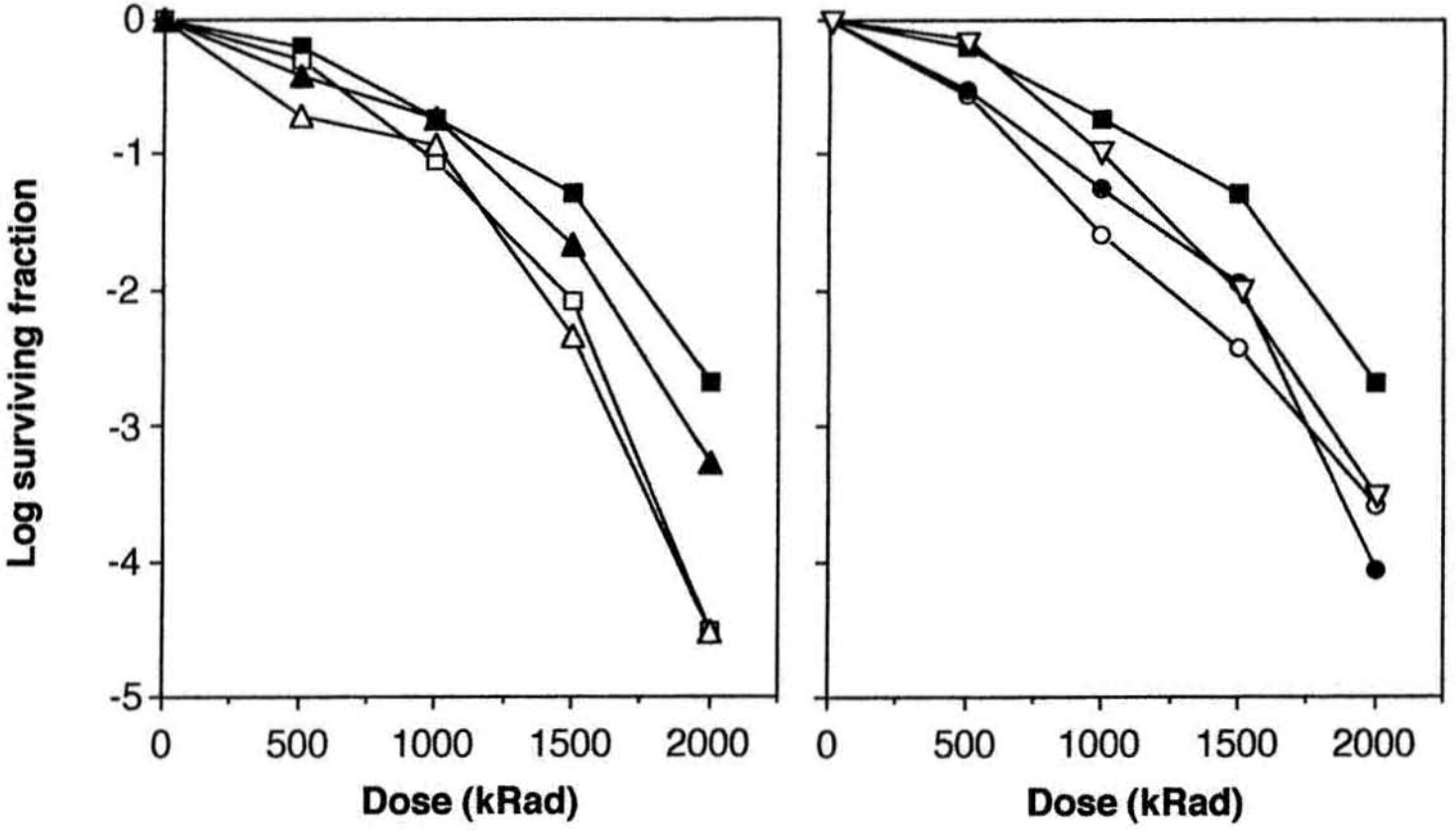


Figure 32.

Cell Survival following Ionizing Radiation Treatment.

Cell Survival following Ionizing radiation treatment



In summary, *E. coli* uvrA is shown to functionally substitute for *D. radiodurans uvrA*. It is apparent that even though the DNA damage level sustained in *D. radiodurans* by various mutagens is in most cases 10X the amount of DNA damage repairable in *E. coli*, the *E. coli* UvrA protein can functionally substitute for *D. radiodurans* UvrA. These findings suggest that *D. radiodurans* UvrA is not the protein responsible for its extreme resistance to DNA damaging agents, but that potentially other DNA repair proteins in this pathway may account for its enhanced efficiency of repair. Future work should focus on identifying the other protein components of the UvrABC repair pathway of *D. radiodurans* and their functions in this pathway.

D. Upstream Region from the *uvrA* Gene.

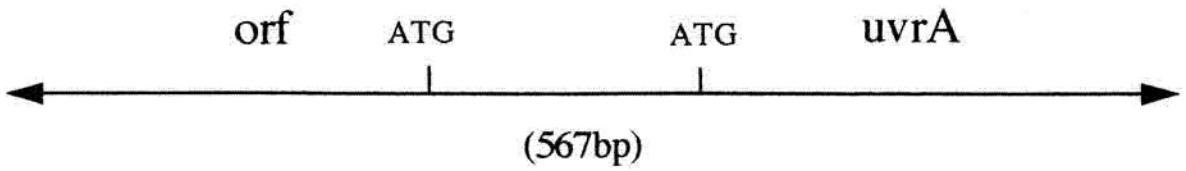
In *E. coli*, single-stranded binding protein (SSB; *ssb*) is found divergently transcribed within a few hundred bases from the *uvrA* gene (Sancar et al., 1981). In fact, these two genes share promoter regions (Sancar et al., 1981). In all other uvrA's identified to date, *Brucella abortus* (Zhu et al., 1993), *S. typhimurium*, and *S. marcescans* (de Vries and Wackernagel, 1993) *ssb* is found divergently transcribed on the opposite strand from *uvrA* (Fig. 33). The exception to this rule is *M. luteus uvrA* (Shiota and Nakayama, 1989) where *ssb* is not found upstream from the *uvrA* gene.

In an effort to identify a possible *ssb* in *D. radiodurans* we sequenced approximately 2,000 bp upstream from the deinococcal *uvrA* (using subclone pHA15b.2 (*Xho* I-*Xho* I)) and did not find any nucleotide sequences homologous to the *ssb* gene of *E. coli* , or other species (Fig.34, 35). However, two open reading frames ranging from 400 to 700 bases were identified (Fig. 35), but at present are not homologous to any known gene by BLAST computer-assisted search.

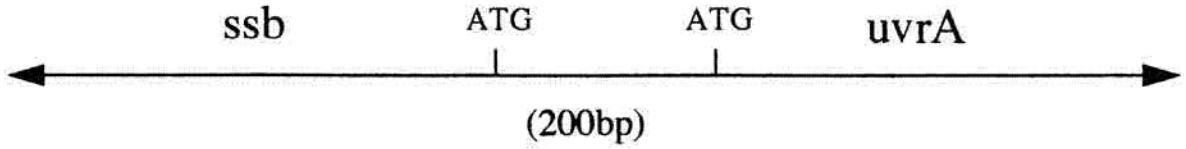
Figure 33.

Comparison of the ssb gene region in various species.

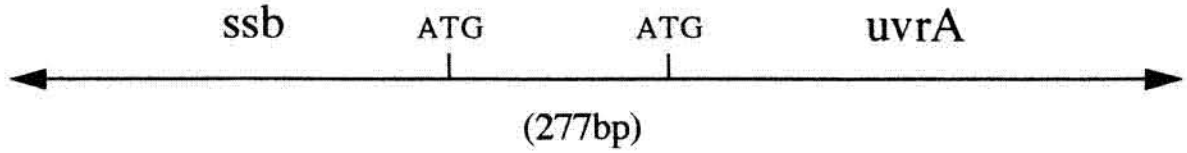
D. radiodurans



E. coli



S. marcescans



B. abortus

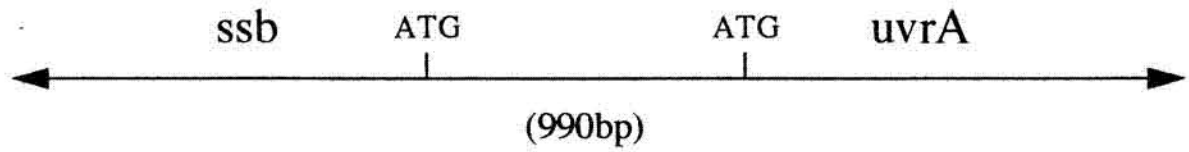


Figure 34.

Map of the *uvrA* gene upstream region. The cloned fragments that were used for sequencing are shown below the chromosomal map. The sizes of the cloned chromosomal fragments are indicated. A, *Acc*I; X, *Xho*I; E, *Eco*RI.

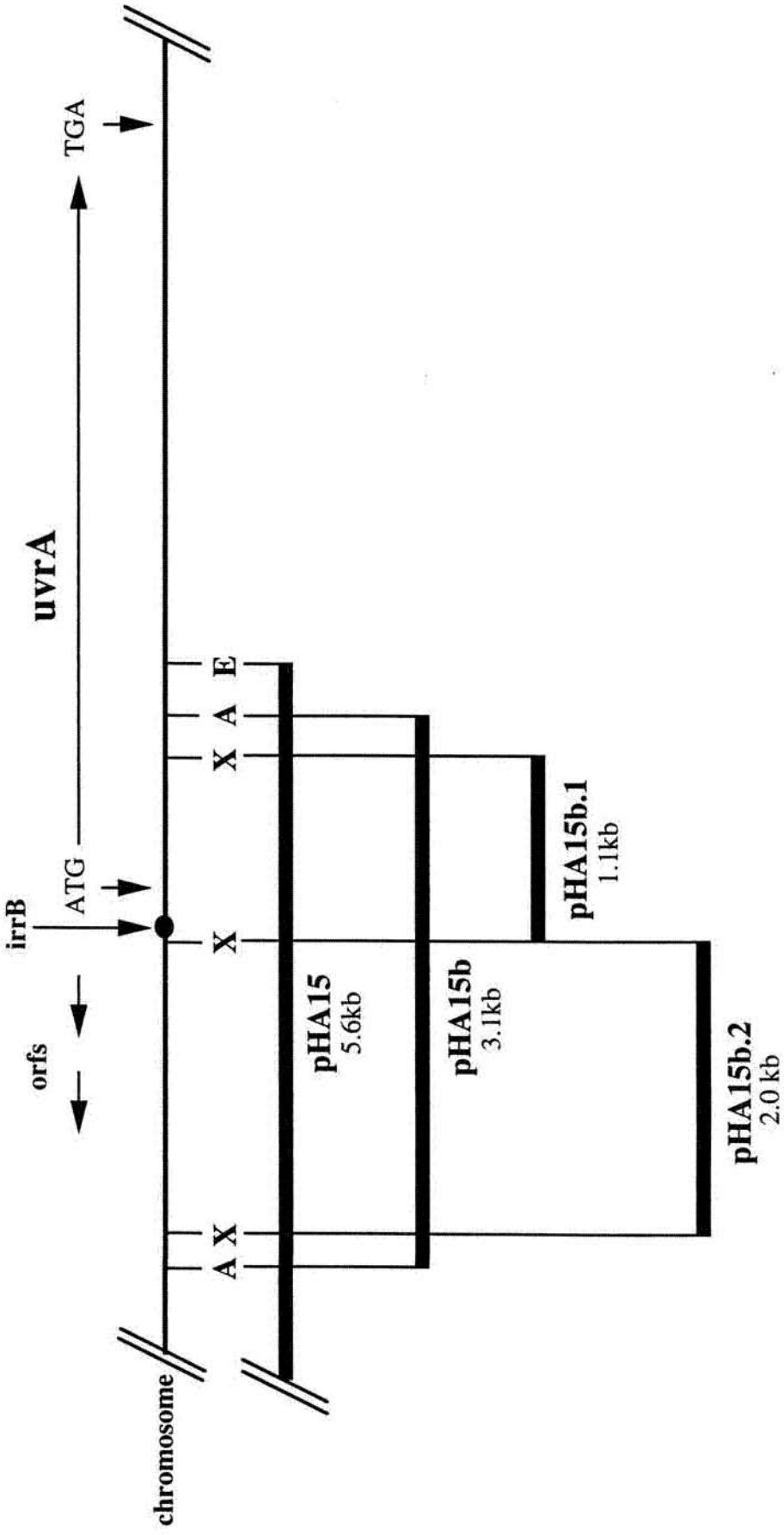

chromosome
A X
X
X A E
irrB
ATG
TGA
uvrA
orfs
pHA15
5.6kb
pHA15b
3.1kb
pHA15b.1
1.1kb
pHA15b.2
2.0 kb

Figure 35.

Panel A. Nucleotide sequence of the pHA15b.2 (*Acc*I to *Xho*I segment). Numbering starts at the first nucleotide of the *Acc*I recognition sequence. **Panel B. Open reading frame analysis of the pHA15b.2 nucleotide sequence.** There are two open reading frames of about 370 and 740 nucleotides in the 6th reading frame of the pHA15b.2 sequence. Neither ORF is homologous to any known sequence as determined by BLAST computer based homology search.

Nucleotide sequence of uvrA upstream region.

```
1
ACCTTCCCTACTGCCGCCTCGGCCAA AATCTTCTTGGACGTTCAGAACGGTGGATT GGTCGGCAAGTGGGAGGAATTTCTCCTGCC    90

TTAACGCCATCCCCATCACCCATCAAC GCAGTGAAAACCATCAACCCCTCATCATCAC CCTGCAACCTCCCGCCGCACATTTTCATCG   180

CCGCCGCCAGTTGCCTCGCGCAAGTC GGCGGGCAGACCAGCGCGCCCGGCCACCAG CAGGCGCACGCCCTCGTCGGGGTCGGCGGC   270

CGCGGGAAATCACTTCGGCGGGCAGC TCGCGCGGCGTCAGAAGGGTTCGGCGCACC CCCGCGTTCGGGTCGGCGGAAAGCTGACGC   360

ACTCGGGCGGCACGTCGGCGCCAGGG CGCCCCCCGGGCACCTCGGCGTCTCGTCGG CGGCGAGGCCGGCCAGTTCGGGGCCGAGGT   450

AGCACGCGAGGATGGTCCGCACGTCC GGTGTCTCGTCGGTCAGCAGGGCTTCGAGG GCGGCGGCGGGCAGCTTCCTCGGCACTCGC   540

TGCAAGTGGAGGTCGGGAATGGGGGG CCTGCTGCGAGGGCAAGAATTGCCGCCGTC GGGCAGGTCGTTGCGTTCGAGCAGTGTCCG   630

ACGGTCCCCGATTCGTCATGAGCGAG GCGTTCCAGCACTTCTGGCGACAGATCAGG ACGGCGGGCTAGGGCCGAGCGAACGTCGGG   720

CGTCGGTTTCGGCGCGGGCGAGCCA CGACTCGGGCACCGTCCAGGCTTGCAGCGC CGCCGAGCGCACCAGCGCGTCCGCACTGGC   810

AGCCACTCGCGCACACTGGCGGGCAG GTCGGTGCGGCGGGCCAGGGTCGCCAGCAC GTCGGGGTCGCCGCCTGTCGCCAGCGAGAG   900

CAGTCGAGCGGCAAATCCAGCCGCCG CGCCACGTTCGCCCGCACCAGGGTGTCGGG TCGGTCACCAGCGTCCGCAGCAGGTCGCCG   990

AGGTCGGGGGCCAGCGCGGTGGCCTTG CGCACGTCGTAGTCCTCATCCTCCGCGAGC TGGCGCAGCAGGGGCTCGGGCAGGTCCGGG  1080

GCGCCACCGCCTCGCGCACCTGCCAC CCGGCGTCCCCCGCCAGCGCCGTGACCCGC TCGGGGGAGAGCCCCGGCCGGGCCGCGAGC  1170

TGCGGACCCCGTAGTCCTCGTGCCGC AGCGCCGAATCCACCACCCAGCCGGGCGCG TCGGGCAGCGCGAGCAGCGCCGTGACCCCC  1260

CAGGAAACGACCCCAGCAGGCCGGGG CGCGCGAGCCTCAGCAGCGGCAATCCCGGA TTGCTCAGCACCTCACGCGGAAACTGAGCG  1350

AGCAGGCCCAGCACCTCGGCGGGGGTG TTGGGGTTGAGCGCCACCTGAGCCCGGACG CGGGCGTCGGGATGCGCCGAGAGGCCGCTG  1440

ACGTCCGCCGGGGCGCGGGGCGAGGTC GCCGCCTCCAGCGCCCCGGCGGTGCCGAGC GCCGTGAGCGTGCGGGGGTCGAGTTCGGAA  1530

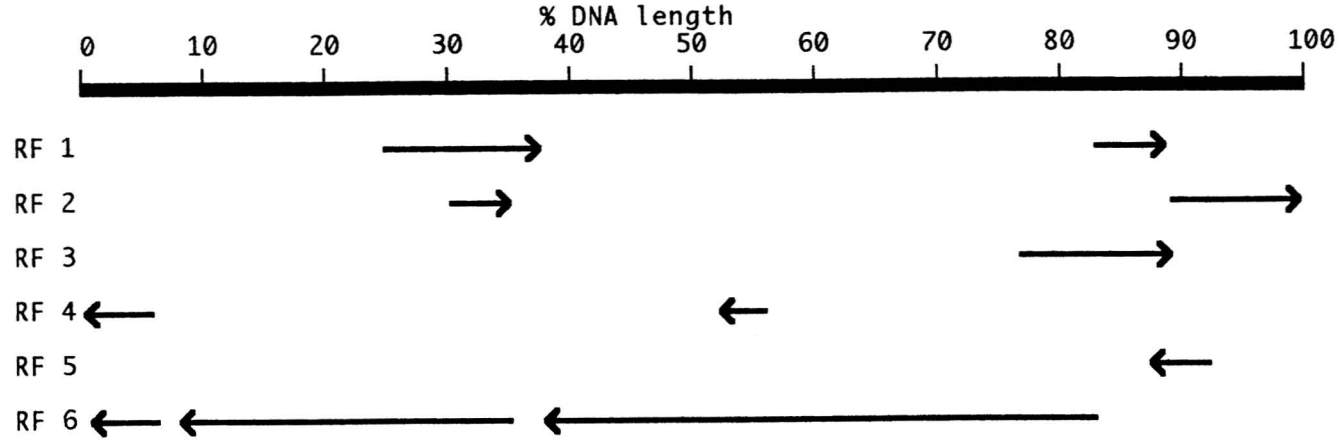
TCATGGGGAGGAGGATAAAGGCCAGT GCCCGCCAGGAAAGCCACAGTTCAGGAAAG CTACAGTTAAGAAAAGGGCCGGCGCTGTCA  1620

CGCTCGTCGCCGCCGCGCAAGCTGA TGGCATGACCTTCGTCGCCCCTTCTGCTCC TCTGAGGCCCAGCGAGCCTGCACCGGGCAT  1710

GTGCGCCAGGCCCACCGCTACCTGCT GGCCCTCGCCCGCCAGCAGCGCCCCGACTG GCGCGACTACCCCCGCGCGGCGCTGCACTG  1800

                                    XhoI
TCGAAGACCGGCAGCTTATCGAGCCGTG CCGGGGTGCACAGGGCCTCGAG                                        1852
```

Open Reading Frames of uvrA upstream sequence.



E. IRS18 Ionizing Radiation Mutant.

In addition to restoring MMC resistance to strain 302, pHA15 was remarkably capable of restoring ionizing radiation resistance to mutant strain IRS18 (Fig. 36). This mutant, IRS18, was generated by MNNG mutagenesis of strain 302 and screened for lack of resistance to ionizing radiation. The IRS18 mutant has the phenotype MMC^S γ^S UV^R, and was developed in the laboratory of John Batistta, Louisiana State University, (New Orleans, Louisiana) (Udupa et al., 1994) . After subcloning pHA15, it was determined that clone pHA15b.1, upon transformation into IRS18, was indeed able to restore radiation resistance (Fig. 37). As mentioned earlier, this subclone, pHA15b.1, also restores MMC resistance to strain 302.

The subclone, pHA15b.1, encodes 262 bases upstream from the translation start site of the uvrA gene. It also includes the first 810 bases at the 5' terminus. A mutation in this region could interfere with the transcription of this gene (*uvrA*) or interrupt the regulatory region 5' to the gene which contains the putative SOS box (which may or may not be functional).

Attempts were made to subclone smaller regions of this gene to determine where the actual mutations exist, but due to time constraints we were unable to complete this task.

Figure 36.

Cell Survival following Ionizing Radiation Treatment. (Reproduced with permission from Battista, 1994)

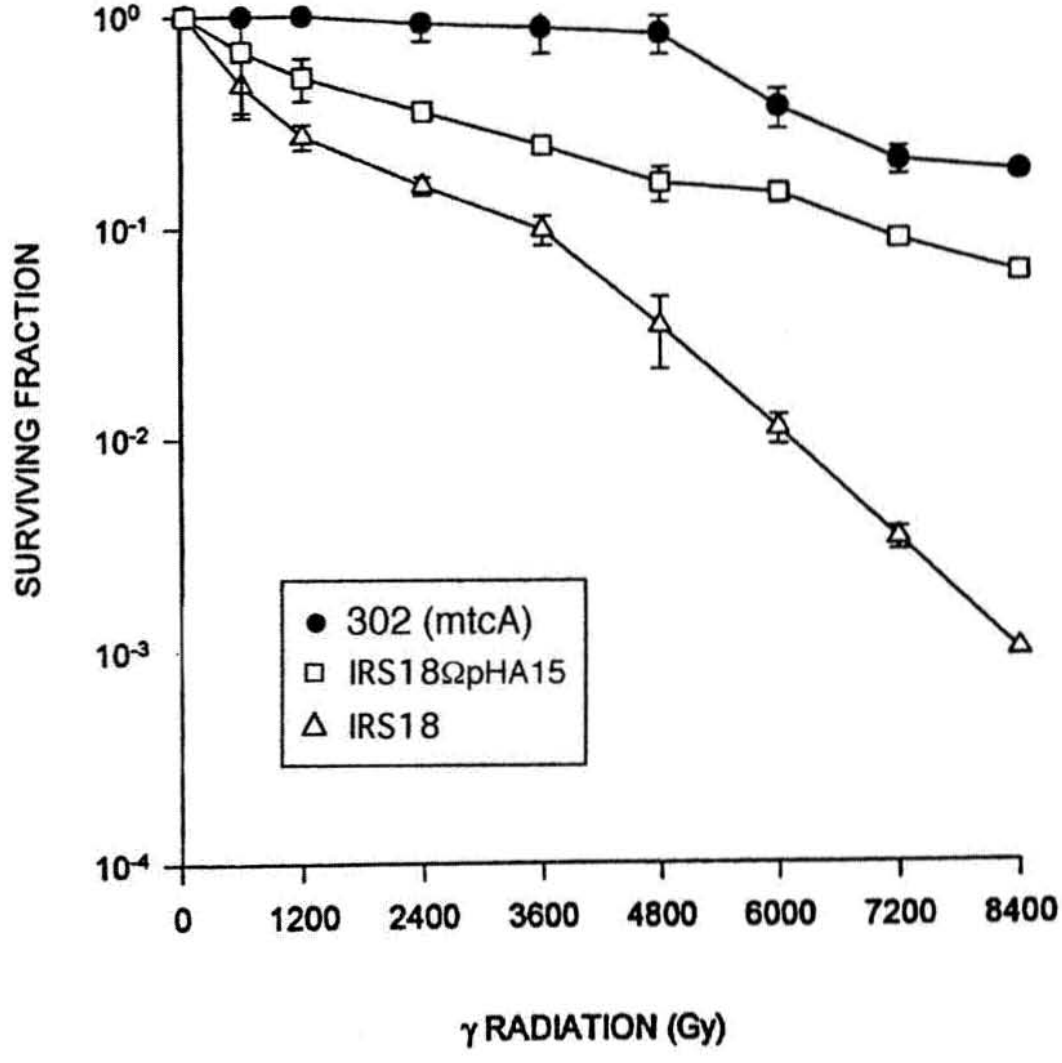
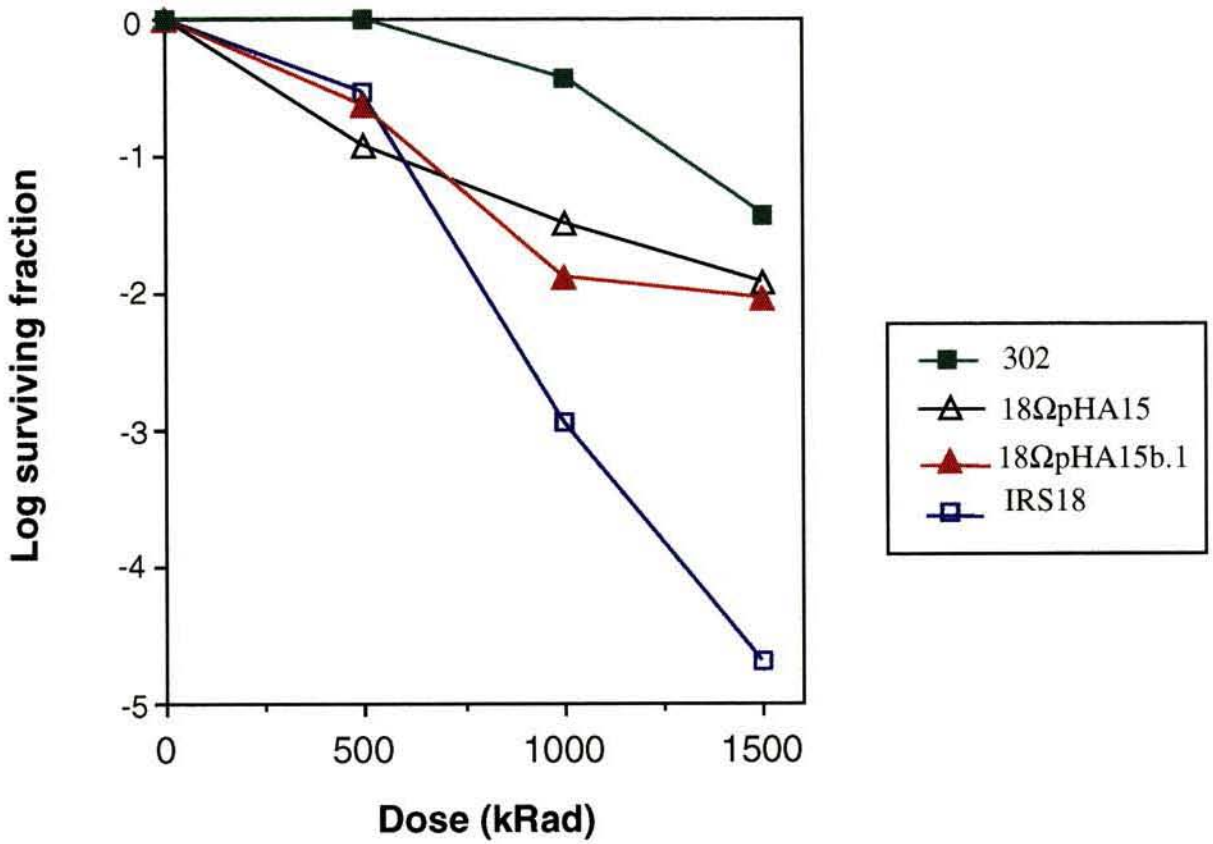

SURVIVING FRACTION
10⁰
10⁻¹
10⁻²
10⁻³
10⁻⁴
0
1200
2400
3600
4800
6000
7200
8400
γ RADIATION (Gy)
302 (mtcA)
IRS18ΩpHA15
IRS18

Figure 37.

Cell Survival following Ionizing Radiation Treatment.

Cell Survival following Ionizing Radiation Treatment



DISCUSSION

General Considerations:

Our interest in understanding the DNA repair process of *D. radiodurans* has led to the identification and characterization of several DNA repair genes in this species, namely homologs of *E. coli recA*, *polA* and most recently by this investigator, *uvrA*. All of these genes demonstrate high degrees of homology at the nucleotide and amino acid level to similar genes found in *E. coli*. These apparent structural homologies, however, do not always imply functional homologies as demonstrated in the case of *uvrA*.

Based on the work in this dissertation, one would at first suspect that few functional differences are seen between *D. radiodurans* and *E. coli uvrA*, but there is still one very important difference between the two proteins. *E. coli uvrA* is under the control of the *recA/lexA* operator and expression of the gene is inducible depending on detection of DNA damage. *D. radiodurans uvrA* is not induced and it is unclear whether a *recA/lexA* regulon is present in *D. radiodurans*. Secondly, we know that *D. radiodurans* possesses two thymine dimer excision repair pathways whereas *E. coli* possesses only one. In addition, resolution of MTC induced cross-links in *E. coli* involves not only the NER (nucleotide excision repair) pathway but also recombination repair. It is probable that recombination repair also occurs in *D. radiodurans* in conjunction with one or both of the excision repair pathways identified. In fact, when *E. coli uvrA* was expressed in uvrA mutant strains of *D. radiodurans* wild-type levels of MTC resistance were observed. This was an unexpected finding since wild-type *E. coli* can repair no more than 2 or 3 cross-links per genome whereas *D. radiodurans* is capable of resolving over 200 MTC-induced cross-links per genome (Kitayama, 1982; Kitayama et al., 1983).

These findings indicate that *D. radiodurans* is able to utilize the *E. coli uvrA* homolog in its pathway for repair of intrastrand cross-links and that there must be another protein(s) responsible for *D. radiodurans'* ability to resolve cross-linked damage so efficiently. A likely candidate for this other protein might be RecA. In fact, the *recA* mutant of *D. radiodurans* is UV

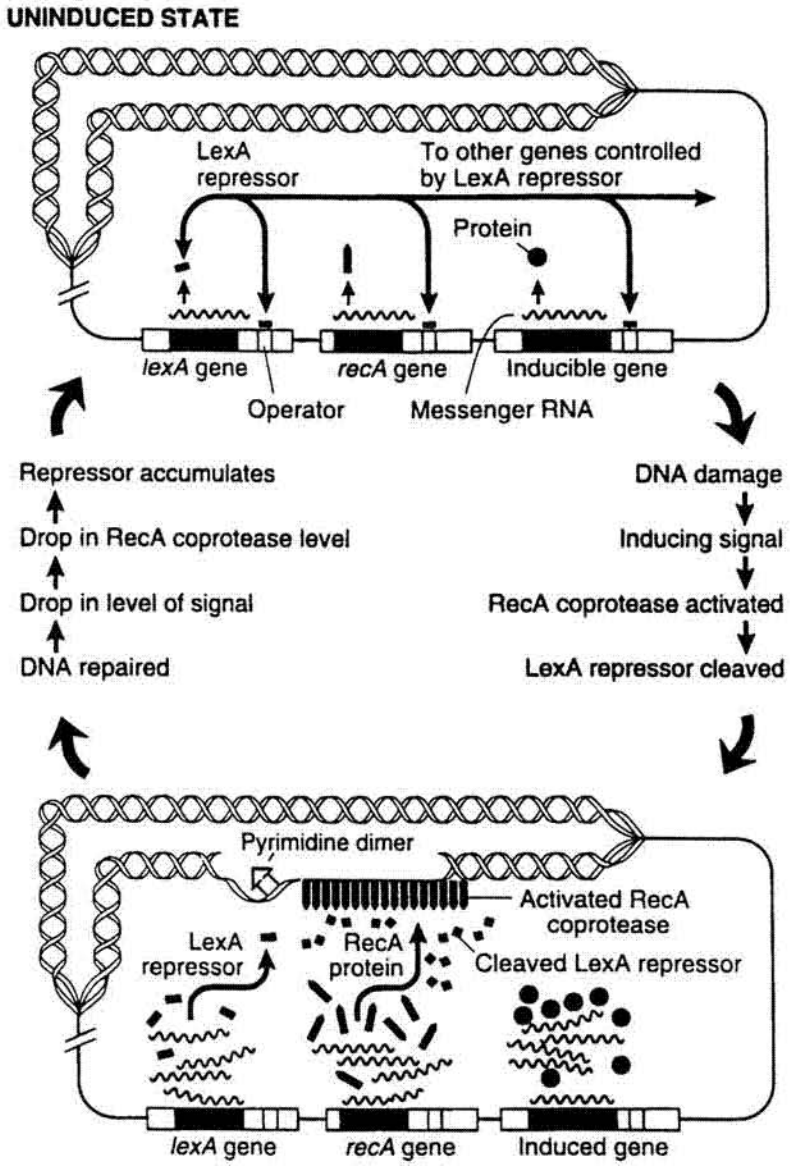
and ionizing radiation sensitive as well as extremely MTC sensitive (Masters et al., 1991). Since the *D. radiodurans recA* homolog was recently identified only limited characterization of the recombination pathway has occurred (Carroll et al., 1996). Future work in this field should include the determination of functional differences between *E. coli recA* and *D. radiodurans recA*.

Besides having a critical role in recombination, RecA also functions as an inducing signal for several DNA repair genes (Fig. 38). In *E. coli*, an inducing signal, normally single-standed DNA, results in increased synthesis of RecA. The RecA protein is then activated and cleaves the repressor, LexA, which occupies the operator sites of various DNA repair genes including *uvrA*. Once cleavage of LexA occurs, expression of these DNA repair genes is induced. As the damaged bases are repaired, the inducing signal is decreased, resulting in decreased activation of recA. As a result, LexA repressor then binds to the operator regions of the DNA repair genes and inhibits expression.

In *D. radiodurans*, this pathway has not been defined. In fact, UvrA is not induced (Evans and Moseley, 1983). However, several proteins are induced in *D. radiodurans* after UV radiation exposure (Hansen, 1980), but the pathway by which this induction occurs has not been identified. Does a LexA-RecA regulon exist in *D. radiodurans*? If one does exist, then what DNA repair genes are induced? Clearly, UvrA expression appears not to be associated with a regulon. Obviously, future work should focus on determining whether the LexA-RecA regulon is present in *D. radiodurans*, as well as identifying the DNA repair genes involved in RecA induction.

Figure 38.

Diagrammatic representation of the mechanism by which the lexA-recA regulon is regulated. (Reproduced with permission from Freidberg et al., 1995)


UNINDUCED STATE
LexA repressor
To other genes controlled by LexA repressor
Protein
lexA gene
recA gene
Inducible gene
Operator
Messenger RNA
Repressor accumulates
Drop in RecA coprotease level
Drop in level of signal
DNA repaired
DNA damage
Inducing signal
RecA coprotease activated
LexA repressor cleaved
Pyrimidine dimer
Activated RecA coprotease
LexA repressor
RecA protein
Cleaved LexA repressor
lexA gene
recA gene
Induced gene
INDUCED STATE

Specific Considerations:

1. Comparison of *E. coli uvrA* and *D. radiodurans uvrA* Genes.

As previously mentioned, *D. radiodurans* and *E. coli uvrA* are about 57% homologous at the amino acid level, with all 5 major motifs conserved. Several interesting features are observed in this sequence. First, the presence of a *lexA* binding domain 5' to the translation start site raises several questions about the regulation of *uvrA* expression in Deinococcus. Is or was *D. radiodurans uvrA* ever under the control of the LexA repressor? Is there a *lexA* gene in Deinococcus, and does it regulate the expression of other DNA repair genes if not *uvrA*?

Previous work by Evans and Moseley (1983) indicate that the gene represented by the 302 mutation (*uvrA*) is able to act in the absence of protein synthesis, suggesting that it is constitutive. However, if the *E. coli uvrA* protein is not inducible in *D. radiodurans*, as shown in this thesis (Fig. 28, 29, 30), the constitutive level is functionally adequate to cope with large amounts of DNA damage. *E. coli* UvrA is a low abundance protein and when induced only increases by a factor of four or five. Is it not possible that *D. radiodurans* UvrA is also induced, but by as low an increase (or less) as *E. coli* UvrA? If this were the case then an induction of *D. radiodurans uvrA* may have been missed by the experimental methods used in earlier experiements (Evans and Moseley, 1983).

If *D. radiodurans uvrA* truly is constitutively expressed, then this implies that there is still a more remarkable component of this pathway which has not been identified. With only about 25 molecules per cell (assuming an equivalent level compared to *E. coli)* how is *D. radiodurans* able to recognize greater than 100 adducts generated by 4NQO treatment? If UvrA protein is in such low amounts then it must be likely that other processes (such as the presence of cell cycle control mechanisms) must be present.

As previously mentioned, future work in this area should include the search for a *lexA*-like gene. If the corresponding operator is functional in *D. radiodurans*, and it is locked into a constitutive level of expression, would changing it back to an inducible state detrimentally effect NER in Deinococcus?

Secondly, the first Zn-binding domain is nearly identical to the *M. luteus* Zn-binding domain in the sense that a histidine may be functioning in the place of the cysteine of *E. coli* uvrA. This suggests that *M. luteus* and *D. radiodurans* are more closely related than *E. coli* and *D. radiodurans.* Is it possible that *D. radiodurans* genomic map is similar to *M. luteus* map? The identification of future genes in *D. radiodurans* may be facilitated by the close observation of the map locations of various DNA repair genes in *M. luteus* as they are discovered.

2. Expression of *E. coli* UvrHomolog in *D. radiodurans*.

From the survival curves shown in this dissertation it is clear that *E. coli* UvrA can functionally replace *D. radiodurans* UvrA. Is it also possible that *M. luteus* UvrA (which is more homologous at the nucleotide level) can just as easily function in place of *D. radiodurans* UvrA? What then accounts for *D. radiodurans* enhanced NER capabilities compared to *E. coli*? It is quite remarkable that *D. radiodurans* can utilize *E. coli* UvrA in Deinococcus cells and repair 10X more MTC-induced DNA damage than *E. coli* is normally capable of repairing. Since there appears to be no great differences between the UvrA's from the various species previously mentioned, it is highly likely that other DNA repair proteins in *D. radiodurans* may possess unusual DNA repair features. It is also likely that *D. radiodurans* unusual two excision repair pathways share the burden of repairing DNA damage in a way that is not observed in other bacteria. Much work needs to be done in this area to identify the other DNA repair genes involved in the excision repair pathways of *D. radiodurans.*

3. Upstream Region from *D. radiodurans* uvrA Gene.

The fact that *D. radiodurans uvrA*, like *M. luteus uvrA* does not possess an ssb gene upstream from its coding sequence is an unepected finding and demonstrated how closely related (phylogenetically) *D. radiodurans* and *M. luteus* must be. It is probably the case that *D. radiodurans* possesses an ssb gene and the identification of this gene will have to await the development of a mutant strain for this gene and further sequencing of the DNA repair genes in this species.

4. Ionizing Radiation Mutant, IRS18.

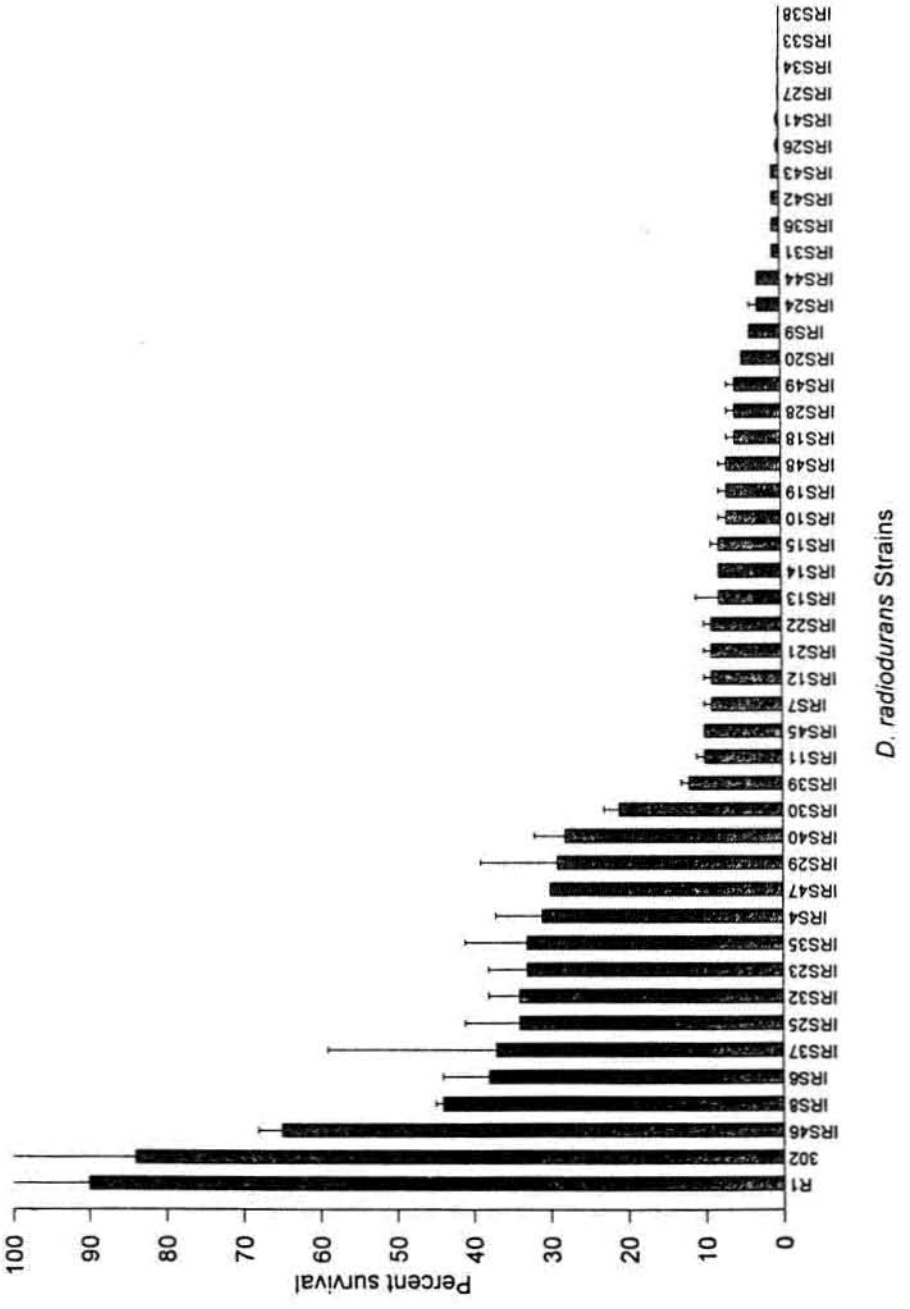
The IRS18 (γ^S UV^R MTC^S) mutant was developed by MNNG mutagenesis of strain 302 (γ^R UV^R MTC^S). MNNG mutagenesis, and as mentioned previously results in increased levels of O^6-alkylguanine and a O^4-alkylthymine. These mutated bases can mispair during semi-conservative DNA synthesis forming mutagenic lesions. Therefore, the IRS18 mutant, may possibly have two point mutations in critical genes which provide γ radiation resistance and MTC resistance. As shown in Fig. 39, the mutation in IRS18 quite profoundly affects its ionizing radiation resistance (Mattimore et al., 1995). As shown in this dissertation, the pHA15b.2 (1.1kb) clone completely restores ionizing radiation resistance as well as MTC resistance to IRS18.

As mentioned, the pHA15b.2 clone includes a short segment 262 base pairs upstream of the *uvrA* translation start site and includes the first 810 base pairs of the 5'-terminus. This mutation, most probably a single base change resulting from MNNG mutagenesis, could be located in the upstream region of the gene which could function as a master regulatory site for several genes. The other option is that the defect may be within the first ATP-binding site of the *uvrA* gene or perhaps anywhere in the 5' terminal region which could potentially result in a frame shift mutation. Mutations in this region of *E. coli* uvrA are known to destroy its DNA binding

function, resulting in a lack of excision of damaged bases (Claassen and Grossman, 1991). Even if this is the case, *E. coli* uvrA has not been shown to function in repair of ionizing radiation induced DNA damage. And if the mutation in *D. radiodurans uvrA* is in fact within the coding region of the gene, repair of ionizing radiation DNA damage would be a novel function for this gene unmasked only in the *irrB* background.. As mentioned previously, *D. radiodurans* does possess novel properties not found in *E. coli* uvrA (such as a lack of induction) and the hypothesis of involvement in ionizing radiation repair is a definite possibility.

Figure 39.

Survival of IRS strains following exposure to γ radiation. (Reproduced with permission from Battista et al., 1994)


Percent survival
100
90
80
70
60
50
40
30
20
10
0
R1
302
IRS46
IRS8
IRS6
IRS37
IRS25
IRS32
IRS23
IRS35
IRS4
IRS47
IRS29
IRS40
IRS30
IRS39
IRS11
IRS45
IRS7
IRS12
IRS21
IRS22
IRS13
IRS14
IRS15
IRS10
IRS19
IRS48
IRS18
IRS28
IRS49
IRS20
IRS9
IRS24
IRS44
IRS31
IRS36
IRS42
IRS43
IRS26
IRS41
IRS27
IRS34
IRS33
IRS38
D. radiodurans Strains

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