THE RESPONSE OF DEINOCOCCUS SP. TO OXIDATIVE STRESS
THE RESPONSE OF RADIATION RESISTANT BACTERIA

DEINOCOCCUS SP. TO OXIDATIVE STRESS

By

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ABSTRACT

Bacteria of the radiation resistant genus *Deinococcus* have a high resistance to the lethal and mutagenic effects of many DNA damaging agents, however, the mechanisms involved in the response of these bacteria to oxidative stress are poorly understood. To investigate antioxidant enzyme responses in *Deinococcus* sp., the types of catalase and superoxide dismutase (SOD) produced by these bacteria were identified by visualization of the enzyme activities on non-denaturing polyacrylamide gels (PAGE) and the growth inhibition of selected strains by various concentrations of hydrogen peroxide and paraquat (a superoxide generating agent) was tested. *Deinococcus* sp. were found to be more resistant to hydrogen peroxide and more sensitive to paraquat than *Escherichia coli* K12. They possess relatively high levels of catalase and exhibit similar electrophoretic patterns on catalase zymograms compared to *E. coli*, but all the tested strains produce only one SOD except *Deinococcus radiophilus* which produces two. The two catalases of *Deinococcus radiodurans* were found to be regulated independently. Cultures of *D. radiodurans*, when pretreated with sublethal levels of hydrogen peroxide, became relatively resistant to the lethal effects of H₂O₂ and exhibited higher levels of catalase than untreated control cultures. The pretreated cells were also relatively resistant to UV- and γ-ray-mediated lethality. These results suggest that *Deinococcus* sp. possess inducible defense mechanisms against the deleterious effects of oxidants and ionizing and
UV radiation. The resistance to the lethal effects of hydrogen peroxide and UV radiation can not be induced by pretreatment with sublethal levels of hydrogen peroxide in *D. radiodurans* rec30 mutant. Effects of manganese and magnesium on the growth and catalase activity of *D. radiodurans* were determined. Homology between *E. coli* catalase and SOD genes and those of *Deinococcus* sp. was tested by Southern blot analysis using previously cloned genes from *E. coli*.
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# TABLE OF CONTENTS

ABSTRACT ..................................... iii

ACKNOWLEDGEMENTS ............................ v

TABLE OF CONTENTS ............................. vi

LIST OF FIGURES ................................ ix

LIST OF TABLES ................................... x

LIST OF ABBREVIATIONS ........................... xi

CHAPTER 1. LITERATURE REVIEW .................... 1

   Introduction .................................... 1
   Characteristics of *Deinococcus* Sp. ............... 3
   Radiation Resistance in *Deinococcus* Sp. .......... 6
      Excision Repair ................................ 7
      Recombination Repair ........................... 8
   Photoreactivation Repair and Inducible DNA Repair ... 9
   Studies on Molecular Mechanisms of Radiation Resistance 10
   Oxygen Radical Toxicity .......................... 12
      Sources of Oxidative Stress ..................... 14
      Damage Caused by Oxidative Stress ............... 16
   Cellular Defense Responses to Oxidative Stress .... 17
      Superoxide Dismutase (SOD) ..................... 18
      Catalase ....................................... 18
      Other Enzymes ................................ 20
   Regulation of Genes Induced by Oxidative Stress .... 21
      Genes Induced by Hydrogen Peroxide ............... 21
      Genes Induced by Superoxide ..................... 23
      Overlap with Other Stresses .................... 25
   Responses of *Deinococcus* Sp. to Oxidative Stress .. 25
CHAPTER 2. MATERIALS AND METHODS .............................................. 28
  Chemicals and Enzymes ......................................................... 28
  Bacteria and Plasmids ........................................................... 28
  Growth Conditions ............................................................... 29
  Effects of Mn(II) and Mg(II) on Growth and Catalase Activity of
  D. radiodurans R1 ................................................................. 29
  Response to Chemical Oxidants .............................................. 30
  Preparation of Cell Extracts ................................................ 30
  Determination of Protein .................................................... 31
  Enzyme Activity Assay .......................................................... 31
  Polyacrylamide Gel Electrophoresis (PAGE) ............................. 32
  Culture Methods for Killing Experiments ................................ 32
  Effect of Hydrogen Peroxide on Catalase Expression ................ 34
  Isolation of DNA ................................................................. 34
  Southern Blotting Analysis .................................................. 35

CHAPTER 3. RESULTS .............................................................. 37
  Effects of Mn(II) and Mg(II) on Growth and Catalase Activity
  of D. radiodurans R1 ............................................................. 37
  Antioxidant Enzymes Produced by Deinococcus Sp. ...................... 38
  Response to Hydrogen Peroxide and Paraquat ............................ 44
  Expression of Catalase in D. radiodurans R1 ............................. 45
  Adaptation of D. radiodurans R1 to Hydrogen Peroxide ............... 50
  Catalase Induction by Hydrogen Peroxide ................................ 52
  Induced Resistance to UV- and γ-Ray-mediated Lethality ............. 55
  Sensitivity of Cultures at Different Growth Stages to UV Radiation . 58
  Catalase and RecA in the Defense of D. radiodurans
    Against H_2O_2-mediated Lethality ...................................... 60
  Response of D. radiodurans rec30 to Hydrogen Peroxide .............. 62
  Hydrogen Peroxide Pretreatment and Protection of D. radiodurans
    rec30 Against H_2O_2- and UV-mediated lethality ................... 62
  Homology Between E. coli Catalase and SOD Genes and
    Those of Deinococcus Sp. ................................................... 67
CHAPTER 4. DISCUSSION ................................................. 69
Antioxidant Enzymes (Catalase and SOD ) and Normal Aerobic Life . 70
The Response of Deinococcus Sp. to Oxidative Stress ............... 71
The Regulation of Catalase in Deinococcus Sp. ......................... 72
Adaptive Response of D. radiodurans to Hydrogen Peroxide ....... 74
Possible Roles of Catalase and RecA in the Defense of
   D. radiodurans Against Hydrogen Peroxide .................... 77
Homology Between E. coli Catalase and SOD Genes and
   Those of Deinococcus Sp. ............................................. 78
Conclusions ............................................................... 79

REFERENCES .......................................................... 81
LIST OF FIGURES

Figure 1. Effects of Manganese on Growth and Catalase Activity of
\textit{D. radiodurans} R1 ................................................. 39
Figure 2. Effects of Magnesium on Growth and Catalase Activity of
\textit{D. radiodurans} R1 ................................................. 40
Figure 3. Electropherogram Showing Catalase Isozymes of
\textit{Deinococcus} Sp. ................................................. 42
Figure 4. Electropherogram Showing SOD Isozymes of
\textit{Deinococcus} Sp. ................................................. 43
Figure 5. Survival of \textit{D. radiodurans} and \textit{E. coli} After
Challenge with H$_2$O$_2$ ............................................. 47
Figure 6. Kinetics of Killing of \textit{D. radiodurans} and \textit{E. coli} by H$_2$O$_2$ 48
Figure 7. Growth and Catalase Expression of \textit{D. radiodurans} ....... 49
Figure 8. Induced Resistance to H$_2$O$_2$ in \textit{D. radiodurans} ........ 51
Figure 9. Induction of Catalase in \textit{D. radiodurans} by H$_2$O$_2$ ....... 53
Figure 10. Electropherogram Showing Induced Catalase by H$_2$O$_2$ .... 54
Figure 11. H$_2$O$_2$-induced Resistance to UV Radiation in \textit{D. radiodurans} 56
Figure 12. H$_2$O$_2$-induced Resistance to $\gamma$-radiation in \textit{D. radiodurans} 57
Figure 13. Sensitivity of Cultures at Different Growth Stages to
UV Radiation .......................................................... 59
Figure 14. Sensitivity of \textit{D. radiodurans} Wild Type (R1) and
rec30 Mutant to UV Radiation ........................................ 61
Figure 15. Inactivation of \textit{D. radiodurans} Wild Type (R1) and
rec30 Mutant by H$_2$O$_2$ ................................................ 63
Figure 16. Induction of Catalase in \textit{D. radiodurans} rec30 by H$_2$O$_2$ .... 64
Figure 17. H$_2$O$_2$-induced Resistance to H$_2$O$_2$ in
\textit{D. radiodurans} rec30 Compared with \textit{D. radiodurans} R1 .... 65
Figure 18. H$_2$O$_2$-induced Resistance to UV Radiation in
\textit{D. radiodurans} rec30 Compared to \textit{D. radiodurans} R1 .... 66
Figure 19. Southern Blot Analysis of Chromosomal DNA of \textit{Deinococcus} sp.
Using pAMkatE22 as the Probe ........................................ 68
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1</td>
<td>Catalase Activity in <em>Deinococcus</em> sp.</td>
<td>41</td>
</tr>
<tr>
<td>Table 2</td>
<td>Sensitivity of <em>Deinococcus</em> sp. to Oxidants</td>
<td>46</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>FUV</td>
<td>far-ultraviolet</td>
</tr>
<tr>
<td>HP</td>
<td>hydroperoxidase</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase or kilobase pair</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>krad</td>
<td>kilorad</td>
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<tr>
<td>mg</td>
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<td>manganese</td>
</tr>
<tr>
<td>NUV</td>
<td>near-ultraviolet</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>Tris</td>
<td>hydroxymethyl aminomethane (C$<em>4$H$</em>{11}$NO$_3$)</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
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<tr>
<td>µl</td>
<td>microliter</td>
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<tr>
<td>µM</td>
<td>micromolar</td>
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CHAPTER 1. LITERATURE REVIEW

Introduction

Bacteria of the genus *Deinococcus*, formerly a part of *Micrococcus* (Murray, 1981), are a group of four species which share high resistance to both the lethal and mutagenic effects of ionizing and ultraviolet (UV) radiation and many DNA-damaging agents (Moseley, 1983). The four species, *Deinococcus radiodurans*, *Deinococcus radiophilus*, *Deinococcus proteolyticus* and *Deinococcus radiopugnans* comprise one of the eight major groups of eubacteria (Fox et al., 1980). Their extremely high radiation resistance, no loss of viability up to doses of 500 krad or 500 Jm⁻² of ionizing or UV radiation, respectively, has made them a particularly useful tool for studying aspects of DNA damage and repair (Moseley, 1983). Most of the work on the radiation resistance mechanisms of *Deinococcus* has focussed on *Deinococcus radiodurans*, the type species of the genus. Its unusually high radiation resistance has been attributed to an effective constitutive DNA repair system based on extensive investigations on radiation-sensitive mutants (Moseley and Laser, 1956; Moseley et al., 1972; Moseley and Copland, 1975; Evans and Moseley, 1983; Moseley and Evans, 1983). Nucleotide excision and recombination repair are the only two mechanisms for the repair of UV light-damaged...
DNA in *D. radiodurans* that have been found (Boling and Setlow, 1966; Moseley *et al.*, 1972; Moseley and Copland, 1975). Evidence for an inducible error-prone DNA repair pathway (SOS system) has not been obtained (Moseley, 1983). Other mechanisms may also be involved in radiation resistance. Since many of the effects of ionizing radiation are oxygen-enhanced (Hollaender *et al.*, 1951a, 1951b; Okazawa and Matsuyama, 1967; Misra and Fridovich, 1976; Myers and Johnson, 1977; Fridovich, 1978), it is possible that oxygen free radicals may play an important role in radiation-mediated toxicity.

Reactive oxygen species including the superoxide radicals ($O_2^\cdot$), hydrogen peroxide ($H_2O_2$), and hydroxyl radicals (OH·) are generated during cellular respiration (Fridovich, 1978). They can also be generated by lethal and mutagenic ionizing radiation (Repine *et al.*, 1981) and near ultraviolet (NUV) (McCormick *et al.*, 1976; Sammartano and Tuveson, 1983; Eisenstark, 1989). These reactive species can oxidize membrane fatty acids initiating lipid peroxidation (Mead, 1976), oxidize proteins (Davies *et al.*, 1987), and damage DNA (Fridovich, 1978; Brawn and Fridovich, 1981). Aerobic-respiring organisms, like *Deinococcus* sp., must therefore possess defense mechanisms to avoid and repair this damage. Enzymes, such as superoxide dismutase and catalase, perform the former function.

*Deinococcus radiodurans* possesses high (relative to *Escherichia coli*) catalase and superoxide dismutase (SOD) activity (Chou and Tan, 1990), so it is useful for studying the oxidative defense mechanisms in radiation-resistant bacteria and the correlation between radiation resistance and high levels of antioxidant enzymes. Although the types
of antioxidant enzymes in these bacteria and the mechanisms involved in the response of these bacteria to oxidative stress are poorly understood, the high catalase and SOD activities may be important in their high radiation resistance. It is necessary to determine the types of antioxidant enzymes in *Deinococcus* sp. and test the response of these bacteria to oxidative stress in comparison with other, extensively-studied bacteria such as *Escherichia coli* and *Salmonella typhimurium*. This may aid in illustrating the mechanisms involved in the defense of *Deinococcus* sp. against oxidative stress. In this review, I will first briefly describe features of *Deinococcus* sp. and then summarize what has been learned to date regarding the radiation resistance of *Deinococcus* sp. Following a review of cellular defense mechanisms to oxidative stress primarily in *E. coli* and *S. typhimurium*, I will discuss the possible mechanisms involved in the response of *Deinococcus* sp. to oxidative stress.

**Characteristics of *Deinococcus* sp.**

*Deinococcus radiodurans* was first isolated by Anderson (1956) from canned meat which was irradiated with a sterilizing dose of γ radiation and was later found in the creek water and soil upstream from the meat plant, as well as on the cattle hair and skin (Krabbenhoft *et al.*, 1965). This strain was named *Micrococcus radiodurans* R1 and is now referred as *Deinococcus radiodurans* R1, the type strain of type species of the genus *Deinococcus*. It was also isolated from other sources, for instance, *Deinococcus*
Deinococcus sp. are aerobic, non-sporeforming, pink-pigmented cocci, usually 0.5-3.5 \( \mu \text{m} \) in diameter, occurring in pairs and tetrads (Murray, 1981). They are oxidase and catalase positive (Murray, 1981). They are Gram-positive, but have an unusual cell wall that includes an outer membrane and fatty acid profiles similar to Gram-negative bacteria (Brooks et al., 1980; Girard, 1971; Work et al., 1968). They share phenotypic characteristics but are distinguishable by a limited number of characteristics. However, since they show no significant DNA homology, they are clearly independent species (Murray, 1986).

Deinococcus sp. are normally grown at 30°C in TGYM broth consisting of 5 g tryptone, 1 g glucose, 3 g yeast extract and 1 g methionine (TGYM) in one liter of distilled water (pH 7.0), with shaking or on TGYM plates containing 1.5% agar. The doubling time of \textit{Deinococcus radiodurans} under these conditions is about 80 minutes and colonies can be counted in two days (Moseley, 1983). They are not heat resistant, and
the thermal maximum is 41°- 43°C (Murray, 1981). There is no appropriate chemically-defined medium (Little et al., 1973; Shapiro et al., 1977) and the chemical requirements for optimal growth remain largely unknown. Manganese has been found to accumulate in large amounts in cells (Leibowitz et al., 1976) and is required for the activity of a DNA repair enzyme (Evans and Moseley, 1985) in *Deinococcus radiodurans*, suggesting that it may play an important role in radiation resistance (Wierowski et al., 1980). Recently, Chou and Tan (1990) have found that the addition of 2.5 μM or higher of manganese to the medium induces at least three rounds of cell division in aging cultures of *Deinococcus radiodurans* IR. *Deinococcus radiodurans* IR is salt-sensitive and responds to 0.8% NaCl added in the medium by showing a growth curve with an unusually long lag phase in which the cells grow and form septa, but do not separate (Chou and Tan, 1991).

Plasmids are found in all strains of the four species of the genus *Deinococcus* viz. *D. radiodurans*, *D. radiopugnans*, *D. radiophilus* and *D. proteolyticus*, except the most-studied strain of the genus, *D. radiodurans* R1. *D. radiophilus* contains three size classes of plasmids while *D. radiodurans* Sark, *D. proteolyticus* and *D. radiopugnans* each contains two (Mackay et al., 1985). The role of the plasmids remains unknown. Plasmids probably do not play an important role in radiation resistance, since no plasmids have been found in *D. radiodurans* R1 in spite of intensive efforts (Moseley, 1983).
Radiation resistance in *Deinococcus* sp.

It is generally assumed that UV and ionizing radiation cause DNA and membrane damage in cells. Among the DNA lesions are pyrimidine dimers, single-strand or double-strand breaks and interstrand cross-links (Kitayama et al., 1983). *Deinococcus* sp. are extremely resistant to UV and ionizing radiation, showing no loss of viability up to doses of 500 J/m² and 500 krad of UV and ionizing radiation, respectively (Moseley, 1983). The survival curves for either UV radiation or γ radiation have a large shoulder, indicating that *Deinococcus* sp. can initially absorb radiation energy with no loss of viability. In other words, these bacteria can accumulate sublethal damage, followed by an exponential loss of viability (Moseley, 1983).

There are some possible explanations for their high radiation resistance. First, *Deinococcus* sp. have an unusual cell wall and membrane structure. This may be important for their radiation resistance, since the repair of DNA damage is dependent on a membrane protein-DNA complex (Montaudon et al., 1987). Membrane composition and lipid fluidity affect the activity of membrane-associated enzymes of DNA repair (Montaudon et al., 1987). The free DNA released into the cytoplasm after irradiation reassociates with the membrane in the case of *D. radiodurans* (Dardalhon-Samsonoff and Rebeyrotte, 1975). It has been suggested that a particular component of the membrane may be an important factor in controlling radiosensitivity of *D. radiodurans* (Montaudon et al., 1987). Secondly, though a correlation between carotenoids and radiation
resistance has not been established, the carotenoids may act as free radical scavengers in *D. radiodurans* (Carbonneau *et al.*, 1989). Therefore, carotenoids may be an indirect contributing factor to radiation resistance, since oxygen free radicals may play an important role in radiotoxicity.

The main reason for the extreme radiation resistance may be that *Deinococcus* sp. have an effective DNA repair system. Moseley and coworkers have examined many facets of the repair capacity of these unusual bacteria and have found that *D. radiodurans* possesses two DNA repair mechanisms: excision repair and recombination repair (for review, see Moseley 1983).

**Excision repair**

There is evidence that *D. radiodurans* possesses an excision mechanism for the release of pyrimidine dimers from DNA (Moseley and Evans, 1983). Two distinct, equally efficient pathways have been found to be involved in this mechanism: one requiring functional *mtcA* and *mtcB* genes, and the other requiring functional *uvsC*, *uvsD* and *uvsE* genes (Moseley and Evans, 1983). The removal of either pathway by mutation does not affect UV resistance, only double mutations result in increased UV sensitivity (Moseley and Evans, 1983). Two UV endonucleases have been found to incise DNA in the response of *D. radiodurans* to pyrimidine dimers caused by UV radiation: UV endonuclease α, the product of *mtcA* and *mtcB* genes, which incises not only pyrimidine dimers, but also mitomycin C, cross-links, bromomethylbenzanthracene adducts and other
alkylation damage, and UV endonuclease $\beta$, encoded by $uvsC$, $uvsD$ and $uvsE$ genes, which incises only pyrimidine dimers (Evans and Moseley, 1983, 1985). Both endonucleases have associated exonuclease activities. These two exonuclease activities can be distinguished by the fact that endonuclease $\alpha$ requires a UV radiation-inducible protein to terminate its activity, while the endonuclease $\beta$ does not (Evans and Moseley, 1983, 1985). The excision repair genes $mtcA$, $mtcB$, $uvsC$, $uvsD$ and $uvsE$ have been cloned (Al-Bakri et al. 1985).

In addition, another two DNA repair enzymes have been found recently in $D.\ radiodurans$ by Masters et al. (1991). The first is a 34 kd protein, which is specific for apurinic/apyrimidinic (AP) sites, called AP endonuclease. Unlike the UV endonuclease $\alpha$ and $\beta$, this enzyme has no activity upon UV-damaged DNA and has no associated exonuclease activity (Masters et al., 1991). The second is a uracil DNA glycosylase, which is active in the presence of EDTA and selectively removes uracil from DNA (Masters et al., 1991). The presence of AP endonuclease and uracil DNA glycosylase suggests that a base excision repair system exists in $Deinococcus radiodurans$. These may be contributing factors to radiation resistance. The details regarding the base excision repair mechanisms remain to be determined.

**Recombination repair**

A second major DNA repair system in $Deinococcus$ sp. may be recombination repair. Evidence for existence of postreplication recombination repair in $D.\ radiodurans$
has been obtained from the studies of a recombination deficient mutant. The mutant was
isolated by Moseley and Copland (1975) and was designated as *D. radiodurans* rec30.
The efficiency of recombination in this mutant was measured by transformation and
shows to be less than 0.01% that of wild type. This mutant is much more sensitive to
UV and ionizing radiation and DNA damaging agents, 15 times as sensitive to UV
radiation, 120 times as sensitive to ionizing radiation and 300 times as sensitive to
mitomycin C as the wild type (Moseley and Copland, 1975). The excision of ultraviolet-
induced pyrimidine dimers is normal (Moseley and Copland, 1975). The other evidence
that *D. radiodurans* possesses an efficient recombination repair is from a comparative
study on sensitivity of *D. radiodurans* and *E. coli* B/r to various mutagens which cause
different kinds of damage to DNA (Sweet and Moseley, 1976). Mutagens can be classed
as repair-dependent and repair-independent according to their effect in repair-deficient
strains of *E. coli* (Sweet and Moseley, 1976). Repair-dependent mutagens, such as UV
radiation, γ-radiation and mitomycin C are mutagenic only in recombination repair-
proficient strains of *E. coli*, while the repair-independent mutagens are effective in
recombination-deficient mutant (Sweet and Moseley, 1976). *D. radiodurans* is resistant
to repair-dependent mutagens but sensitive to repair-independent mutagens (Sweet and
Moseley, 1976).

**Photoreactivation repair and inducible DNA system**

Photoreactivation repair of pyrimidine dimers has not been observed in *D.
radiodurans. When the cells exposed to photoreactivating wavelengths of light following UV irradiation, their survival does not appear to be affected (Moseley and Laser, 1965). It may be argued that a photoreactivating enzyme is present, but this has not been demonstrated, since the repair of UV-induced damage in the absence of light is very efficient. However, if present, an increase in survival of UV-irradiated sensitive mutant, like D. radiodurans rec30, should occur. In fact, an increase in survival of mutant rec30 does not occur under these conditions (Moseley, 1983).

Evidence for an inducible error prone repair pathway (SOS system) has not been obtained for Deinococcus radiodurans and its repair of UV radiation-induced damage is error free (Moseley, 1983). The SOS system was first found present in E. coli and clearly postulated by Defais et al. (1971). It is the largest, best-understood DNA damage inducible system to date. The expression of the genes of this system is under the control of RecA and LexA proteins. LexA acts as the repressor of many genes including recA and lexA. When DNA is damaged or its replication is inhibited, RecA is activated immediately. The activated RecA cleavages LexA and the genes controlled by LexA are expressed. As the cell recovers, RecA is no longer in an activated state and LexA is no longer cleaved. This, in turn, results in the repression of the associated genes (for more details, see Walker, 1987)

Studies on molecular mechanisms of radiation resistance

Many efforts to illuminate molecular mechanisms of radiation resistance of
Deinococcus sp. have been made. Al-Bakri and co-workers (1985) have cloned DNA repair genes of *D. radiodurans* into *E. coli*. This suggests that it may be possible to express other genes in *E. coli*. The radiation resistance gene(s) might be cloned into *E. coli*. This has been done in two different laboratories (Dalrymple *et al.*, 1989; Barrows, 1989). Both groups constructed *D. radiodurans* DNA library in *E. coli* and screened for radiation resistant *E. coli*. They have isolated some clones that showed increased radiation resistance (Dalrymple *et al.*, 1989; Barrows, 1989) and the increased resistance have been correlated to the presence of a 3kb insert from *D. radiodurans* (Dalrymple *et al.*, 1989). However, this conclusion is not consistent with their later studies. Their further attempts to isolate and characterize the responsible *D. radiodurans* DNA fragment(s) in the clones failed and the plasmids recovered from the radiation resistant *E. coli* transformants failed to confer radiation resistance on naive *E. coli* (Barrows *et al.*, 1991). It is possible that their radiation selection may not reflect selection of plasmid-encoded function but selection of radiation resistant *E. coli*. Another reason may be that *D. radiodurans* DNA fragment(s) encoding radiation resistance was (were) not stable when cloned in *E. coli*.

The genetic and molecular mechanisms responsible for radiation resistance have not been identified, in part because genetic techniques applicable to *Deinococcus* species are limited. *D. radiodurans* is the only member of the genus naturally transformable by homologous DNA (Moseley, 1983). *Deinococcus* sp. are not closely related to any genetically well-understood bacteria. There are no other applicable genetic techniques,
such as conjugation, protoplast fusion, transduction or transposons for introduction of genetic markers. Recently, a method that uses transformation of *D. radiodurans* SARK to generate random chromosomal gene fusions with *lacZ* has been reported by Lennon and Minton (1990). Several of these gene fusions have been isolated and are inducible by the DNA damaging agent - mitomycin C. Some of these gene fusions have been cloned (Lennon and Minton, 1990) and shown to hybridize with numerous fragments of genomic DNA (Lennon *et al.*, 1991).

In summary, *D. radiodurans* has an effective constitutive DNA repair system that is important for radiation resistance. However, the molecular mechanisms that play a direct role in radiation resistance are not understood. Attempts to clone the genes responsible for radiation resistance have, thus far, met with failure. It may be possible that the high radiation resistance of *Deinococcus* sp. is due to a combination of several mechanisms including the DNA repair system. Oxidative defense systems may also be involved, since ionizing radiation can exert lethality through the generation of reactive oxygen species (Repine *et al.*, 1981).

**Oxygen radical toxicity**

All aerobic organisms, like *Deinococcus* sp. must defend themselves from the toxic byproducts of oxygen metabolism. During respiration, in addition to the complete four electron reduction of molecular oxygen to water, reactive species can be generated.
They are superoxide radicals (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (OH$^-$). These reactive forms of oxygen can oxidize membrane fatty acids initiating lipid peroxidation, oxidize proteins, and damage DNA. Enteric bacteria produce several enzymes, such as superoxide dismutase and catalase to protect themselves from this oxidative damage.

\[
\begin{align*}
\text{SOD} & \quad \text{HP} \\
4H^+ + 4O_2^- & \rightarrow 2O_2 + 2H_2O_2 \rightarrow 2H_2O + 3O_2
\end{align*}
\]

As shown in the above figure, superoxide dismutase (SOD) catalyses the conversion of superoxide anion to hydrogen peroxide, which in turn is removed by catalase (or hydroperoxidase (HP)) which converts it to water plus oxygen and peroxidases which reduce it to water (Fridovich, 1978). Efficient removal of the first two intermediates of oxygen reduction, O$_2^-$ and H$_2$O$_2$, will prevent the formation of OH$, since hydroxyl radicals (OH$^-$) are formed by the two intermediates reacting together (Imlay and Linn, 1988; Imlay et al., 1988). In addition, other enzymes such as exonuclease III, RecBC and RecA proteins appear to play an important role in repairing oxidative damage (Imlay and Linn, 1988).

*Deinococcus* sp. to oxidative stress are as yet poorly understood. To understand the
responses of *Deinococcus* sp. to oxidative stress, it is necessary to understand the oxygen radical toxicity and cellular defense mechanisms found in other bacteria, such as *E. coli* and *S. typhimurium*.

**Sources of oxidative stress**

"Oxidative stress can be functionally defined as an excess of prooxidants in the cell" (Farr and Kogoma, 1991). Reactive oxygen species arise from a variety of intracellular and extracellular sources. First of all, they are generated as by-products of normal aerobic metabolism. During aerobic growth, aerobic organisms use oxygen as the final electron acceptor in the electron transport chain and the electron(s) from nutrients are finally transferred to oxygen. Subsequently, oxygen is normally reduced to water. However, consecutive univalent reductions of oxygen produce oxygen species with reactive properties, including superoxide anions (O$_2^-$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (OH·). The following figure shows the steps of univalent reduction of O$_2$.

\[
\begin{align*}
O_2 & \rightarrow O_2^- \rightarrow H_2O_2 \rightarrow H_2O + OH^- \rightarrow H_2O
\end{align*}
\]

The major sources of superoxide in *E. coli* have been found to be reactions mediated by NADH dehydrogenase, succinate dehydrogenase and D-lactate dehydrogenase (Imlay and Fridovich, 1991). The glutathione reductase, which uses
NADH as an electron source, is thought to be capable of producing significant amounts of superoxide anions (Massey et al., 1969; Imlay and Fridovich, 1991). The other source of superoxide anions is autoxidation of several cellular constituents such as ubiquinols, catechols and thiols. These cellular components are easily reduced to semiquinones, which in turn reduce $O_2$ to $O_2^-$ (Farr and Kogoma, 1991). In addition, the reduced transition metals in the cells can produce $O_2^-$, arising from donating a single electron to $O_2$ (Farr and Kogoma, 1991).

Hydrogen peroxide is generated by spontaneous and SOD-catalyzed dismutation of $O_2^-$. Other sources of $H_2O_2$ are several oxidases, such as D-amino acid oxidase (Farr and Kogoma, 1991).

The reaction of $H_2O_2$ and reduced iron is considered to be a significant source of $OH^-$. This reaction is referred as the Fenton reaction (Cadenas, 1989):

$$H_2O_2 + Fe^{2+} + H^+ \rightarrow OH^- + H_2O + Fe^{3+}$$

Active oxygen species may be produced by other means, such as ionizing radiation, near-ultraviolet radiation, certain oxidation-reduction drugs, like paraquat. $H_2O_2$ and $OH^-$, for example, can be generated from near-UV radiation and radiolysis of water by $\gamma$ radiation, respectively (Eisenstark, 1989; Repine et al., 1981).

Of the active oxygen species, the $OH^-$ is an extremely powerful oxidant that can react with most cellular constituents, such as DNA, RNA, membrane lipids and proteins.
at diffusion-limited rates (Singh and Singh, 1982). The $O_2^-$ and hydrogen peroxide are assumed to damage macromolecules by generating $OH^-$ in the Fenton reaction (Cadenas, 1989).

**Damage caused by oxidative stress**

Reactive oxygen species cause a great deal of damage to macromolecules *in vivo* and *in vitro*, including damage to DNA, oxidization of proteins and lipids. DNA damage is considered to be the major damage caused by oxidative stress, since strains with mutations in DNA repair genes are hypersensitive to oxidants (Imlay and Linn, 1988). An attack on a DNA molecule by reactive oxygen species results in numerous types of DNA lesions - breaks in one or both strands of the DNA, sugar fragmentation and missing or altered bases, like thymine glycol. Such damage can block replication by DNA polymerase and transcription by RNA polymerase or cause misreading, with the result that truncated or abnormal proteins are produced.

The second damage produced by oxidative stress is membrane damage. These kinds of damage may be from either lipid damage or protein damage. Interaction between reactive oxygen species and lipids leads to the initiation of lipid peroxidation (Mead, 1976). As a result, essential membrane-situated activities, such as energy production or nutrient uptake, are disrupted. Peroxidation intermediates and end products are also mutagenic (Chio and Tappel, 1969; Segerback, 1983; Summerfield and Tappel, 1983). Moreover, oxidative stress can cause alterations of amino acids
(Stadtman, 1986). These alterations generally inactivate enzymes, and the oxidized proteins are hypersensitive to degradation (Storz et al., 1990b).

**Cellular defense responses to oxidative stress**

As discussed above, though reactive oxygen species are toxic to cells, they are naturally produced in aerobic cells by intracellular and extracellular sources. Therefore, aerobic organisms must maintain a strong defense against the threat. In bacteria, there are two separate systems that protect cells from oxidative damage. First, there are scavenging enzymes, including catalase, superoxide dismutase and alkylhydroperoxide reductase, which reduce reactive oxygen species (Storz et al., 1990b). Second, the cell possesses repair enzymes, such as exonuclease III and endonuclease IV, that repair damaged DNA (Storz et al., 1990b). Oxidatively stressed, bacteria may respond by eliminating reactive oxygen species with a combination of the preexisting enzymes, meanwhile turning on the genes involved in the response to oxidative stress to synthesize more defense activities. In this section, I will describe some important defense enzymes in *E. coli* cells and discuss the regulation of the genes induced by oxidative stress to understand how a bacterial cell responds to oxidative stress.

Three superoxide dismutases (SODs) (an iron-containing SOD (FeSOD), a manganese SOD (MnSOD) and a hybrid enzyme composed of both Fe- and Mn-containing subunits), and two catalases (HPI and HPII) are produced in *Escherichia coli*. 
Superoxide dismutase (SOD)

Both MnSOD and FeSOD are small metallo-proteins with a molecular weight of about 20 kd and are able to catalyze the dismutation of $O_2^-$ to hydrogen peroxide and oxygen. The FeSOD is produced constitutively in both aerobic and anaerobic conditions, whereas MnSOD is not normally produced in anaerobic condition (Dougherty et al. 1978; Moody and Hassan, 1984). MnSOD can be induced by the superoxide anion and can also be induced by redox compounds such as paraquat which generates $O_2^-$ under aerobic, but not anaerobic conditions (Hassan and Fridovich, 1980; Hassan and Moody, 1984, 1987). FeSOD and MnSOD are encoded by genes sodB and sodA, respectively. The sodA and sodB genes have been cloned (Sakamota and Touati, 1984; Touati, 1983) and sequenced (Takeda and Avila, 1986; Carlioz et al., 1988).

The molecular regulation of SODs has been studied by Pugh and Fridovich (1985) and Moody and Hassan (1984) and many others. It is suggested that transcription is inhibited by an active ferrous iron-containing repressor protein that is inactive when the iron is in a ferric state or when the protein is stripped of iron. Touati (1988), using protein and operon fusions between the MnSOD gene (sodA) and lacZ suggested that MnSOD is regulated at both the transcriptional and posttranscriptional levels.

Catalase

The two catalases in *E. coli*, a bifunctional catalase-peroxidase HPI and a monofunctional catalase HPII, are quite different from each other and from the typical
catalase (bovine) which is a tetramer of 65 kd subunits and contains four protoheme IX groups. HPI is active as a tetramer of 81 kd subunits and contains two protoheme IX groups (Claiborne et al., 1979). HPII is found to be a hexamer of 93 kd (Loewen and Switala, 1986) and contains six heme d-like groups (Chiu et al., 1989). Moreover, they are induced by different stimuli. HPI is produced under both anaerobic and aerobic conditions and is induced by hydrogen peroxide (Loewen et al., 1985a). The \textit{katG} gene, encoding HPI, has been shown to be under the control of the \textit{oxyR} regulon which responds to oxidative stress (Christman et al., 1985; Morgan et al., 1986). In contrast, HPII synthesis is induced by oxygenation and by the onset of the stationary phase (Loewen et al., 1985a) and might be affected by intracellular pH (Schellhorn and Hassan, 1988). The synthesis of HPII is under the control of two genes, \textit{katE} and \textit{katF}.

The \textit{katG}, \textit{katE} and \textit{katF} genes have been characterized by Loewen's group. The \textit{katG} and \textit{katE} are unlinked, mapping at 89.2 (Loewen et al., 1985b) and 37.2 (Loewen, 1984) min, respectively. The \textit{katF} gene mapps at 59.0 min (Loewen and Triggs, 1984) and is required for expression of \textit{katE}, but not \textit{katG}. Schellhorn and Hassan (1988) suggested that \textit{katE} is the structural gene of HPII and KatF plays a regulatory role. The \textit{katF} gene has been cloned (Mulvey et al., 1988) and sequenced (Mulvey and Loewen, 1989). The sequence similarity between the KatF protein and known sigma transcription factors also suggests that KatF acts as a positive effector of \textit{katE} (Mulvey and Loewen, 1989). The \textit{katE} gene has been sequenced (von Ossowski et al., 1991) and homology studies reveal that HPII has sequence similarity to the common bovine catalase. The
**katG** has been cloned and sequenced and reveals no resemblance to any known catalase sequences (Triggs-Raine et al., 1988).

**Other enzymes**

*E. coli* and *S. typhimurium* possess alkyl hydroperoxide reductase that is encoded by *abpC* and *abpF* and has the ability to reduce hydroperoxides such as thymine hydroperoxide and linoleic hydroperoxide to their corresponding alcohols (Jacobson et al., 1989). A glutathione reductase (encoded by *gorA* gene) may protect cells from toxic oxidants by maintaining a pool of reduced glutathione. The latter can maintain the reduced state of cellular protein (Storz et al., 1990b).

In addition to the defense enzymes that break down oxidants, several DNA repair enzymes appear to play an important role in repairing damage caused by oxidants. Exonuclease III (encoded by *xthA*) and endonuclease IV have been shown to remove replication blocks from 3' termini of oxidized DNA in *E. coli* (Demple et al., 1986). Other DNA repair enzymes including endonuclease III, DNA polymerase I (*polA*), and the excision nuclease and exonuclease associated with *uvrABC* and *recBC*, respectively, are also important for a defense against oxidative stress. Their roles in protection against oxidative stress were illuminated by the findings that strains with mutations in genes encoding these activities are sensitive to hydrogen peroxide and other oxidants (Imlay and Linn, 1988). The *recA* gene is a regulator of the SOS response (induced by many types of DNA damage) and strains with mutations in *recA* gene are sensitive to hydrogen
peroxide (Imlay and Linn, 1988).

**Regulation of genes induced by oxidative stress**

Many of the genes that are required for the defense against oxidative stress in *E. coli* and *S. typhimurium* have been identified. They belong two distinct regulons. Bacteria respond to oxidative stress by quickly turning on one or both of the two regulons.

**Genes induced by hydrogen peroxide**

When the offending oxidant is hydrogen peroxide, cells induce up to 30 proteins (Morgan *et al.* 1986; VanBogelen *et al.*, 1987). Eight in *E. coli* and at least nine in *S. typhimurium* of the induced proteins are positively regulated by the locus, *oxyR*, so the group of the genes encoding these proteins is called the *oxyR* regulon. This regulon was delineated by isolation of an *oxyR* deletion mutant of *S. typhimurium* which fails to induce these proteins (Christman *et al.*, 1985). The *E. coli* *oxyR* gene has been cloned and the nucleotide sequence has been determined (Christman *et al.*, 1989; Tao *et al.*, 1989). The gene product, OxyR, is a 34 kd protein which shares significant homology with the LysR family - a family of bacterial regulatory proteins (Henikoff *et al.*, 1988). The regulation of genes encoding catalase, alkyl hydroperoxide reductase, and other enzymes by OxyR is at the transcriptional level (Morgan *et al.*, 1986). Footprinting analysis with purified OxyR has demonstrated that the OxyR binds to the promoters
upstream from the hydrogen peroxide-inducible genes including *katG*, *ahpCF* and *oxyR* to activate the transcription of the genes (Storz *et al.*, 1990a; Tartaglia *et al.*, 1989). This suggests that in response to hydrogen peroxide, OxyR acts to activate the transcription of a family of hydrogen peroxide-inducible genes.

To show how an oxidative stress signal is transduced to OxyR protein, Storz *et al.* (1990a) prepared OxyR protein under both oxidizing and reducing conditions and determined the ability of OxyR protein to activate the transcription of the *oxyR*-regulated *ahpCF* and *katG* genes *in vitro*. They found that OxyR prepared under the oxidizing conditions binds the gene promoters and activates the gene expression, while OxyR prepared under reducing conditions can bind the promoters of the genes but can not activate the gene expression. However, it can be readily converted to an active form by exposure to air (Storz *et al.*, 1990a). These findings suggest that when hydrogen peroxide enters cells, OxyR becomes oxidized, and the oxidized form of the protein activates the transcription of genes associated with the hydrogen peroxide challenge. As a result, cells acquire resistance to hydrogen peroxide stress. Therefore, cells pretreated with sublethal levels of hydrogen peroxide become relatively resistant to subsequent killing by lethal levels of hydrogen peroxide (Demple and Halbrook, 1983; Christman *et al.*, 1985). During the course of H$_2$O$_2$ pretreatment, DNA repair enzymes may be induced as well. This idea is supported by the finding that H$_2$O$_2$ damaged-lambda phage have a higher plating efficiency on pretreated cells than on naive cells (Demple and Halbrook, 1983). Since lambda phage injected naked DNA into the host cells, increased
plating efficiency indicates an increased repair capacity for the DNA damaged by hydrogen peroxide.

Along with oxyR, the other regulon called oxoR regulon is induced in response to a hydrogen peroxide (Ahern, 1991). The so called later genes involved in the hydrogen peroxide regulon are thought to be controlled by this gene product (Ahern, 1991).

**Genes induced by superoxide**

The other defense regulon, called soxRS, functions, when the offending oxidants are superoxide-generating agents. As in the response to hydrogen peroxide, *E. coli* induces about 40 proteins in response to superoxide-generating agents (Greenberg and Demple, 1989; Walkup and Kogoma, 1989; Greenberg *et al.*, 1990). Nine of these proteins are positively regulated by the locus soxR (superoxide response) at the transcriptional level (Greenberg *et al.*, 1990). At least three of them have demonstrable antioxidative roles: Mn-containing superoxide dismutase destroys superoxide radicals, endonuclease IV repairs radical-induced DNA damage in DNA, and glucose-6-phosphate dehydrogenase produces NADPH (Greenberg *et al.*, 1990). Therefore, pretreatment of *E. coli* cells with nonlethal levels of superoxide-generating agents, such as plumbagin, paraquat and menadione increases resistance to lethal doses (Farr *et al.*, 1985; Greenberg and Demple, 1989).

Activation of the soxRS regulon also increases resistance to many antibiotics including chloramphenicol, tetracycline, ampicillin and nalidixic acid. This may be due
to the decreased synthesis of the OmpF outer membrane protein (Greenberg et al., 1990).


How the soxRS regulon senses the superoxide stress and transduces the signal into transcriptional activation is not completely understood. However, it is different from the oxyR regulon. Cells pretreated with hydrogen peroxide display enhanced resistance to hydrogen peroxide but not to plumbagin (Farr et al., 1985). Similarly, pretreatment with plunbagin leads to an increased resistance to plumbagin but not to hydrogen peroxide (Farr et al., 1985). Proteins induced by the two different kinds of oxidants are different (Walkup and Kogoma, 1989). Regulation of soxRS must be more complex than oxyR. Recently, Amabile-Cuevas and Demple (1991) have proposed an elaborate two-stage
regulatory system for the \textit{soxRS} regulon. In their model, SoxR protein acts as a sensor, while SoxS protein acts as a transducer. First, the cellular signal from superoxide activates preexisting SoxR protein. Activated SoxR then triggers expression of the \textit{soxS} gene, with the resulting increase in SoxS, which in turn activates the expression of the regulon genes.

\textbf{Overlap with other stresses}

Some of the proteins induced by hydrogen peroxide or superoxide, including some DNA repair enzymes, are also induced by heat shock, starvation, and other types of stress. The SOS response, which is induced following serious damage to bacterial cell DNA, may be important in the defense against oxidative stress as well.

\textbf{Responses of \textit{Deinococcus} sp. to oxidative stress}

Since UV and \(\gamma\)-ray radiation can exert deleterious effects through reactive oxygen intermediates, testing of the response of \textit{Deinococcus} sp. to oxidative stress is important for understanding of the defense mechanisms involved in radiation resistance.

The first attempt to investigate oxygen defense mechanisms in \textit{Deinococcus} sp. was testing of the role of carotenoids in \textit{Deinococcus} sp. Of the lipid components, the carotenoids have been found to effectively quench singlet oxygen (Foote, 1968) and oxygen free radicals (Burton and Ingold, 1984). They are possible biological
antioxidants against reactive oxygen species and may be involved in defense mechanisms as free radical scavengers. *Deinococcus* sp. are red-pigmented and have carotenoids. Carbonneau *et al.* (1989) used *Deinococcus radiodurans* as a model to gain further insight into the quenching of OH radicals by carotenoids. They compared the action of H$_2$O$_2$ on the parental and two mutant strains, i.e., a red pigmented and a colourless one. They found that the red pigmented bacteria are relatively resistant to H$_2$O$_2$ action, whereas the colourless strain is significantly more sensitive and this sensitivity is dose-dependent.

Two catalases (catalase A and catalase B) and three superoxide dismutases (FeSOD, MnSOD and hybrid (Mn-Fe) SOD) are produced by *D. radiodurans* IR (Chou and Tan, 1990). Addition of 2.5 $\mu$M or higher Mn(II) to stationary-phase cultures of *Deinococcus radiodurans* IR results in the induction of cell division and an increase in superoxide dismutase and catalase activity (Chou and Tan, 1990). However, the Mn-induced cells exhibit increased sensitivity to UV radiation and $\gamma$ radiation (Chou and Tan, 1990). This requires further study.

Although *Deinococcus* sp. are extremely resistant to the lethal effects of far-ultraviolet radiation (FUV, 254 nm), *D. radiodurans* has been found to be highly sensitive to killing by near-ultraviolet radiation (NUV, 300-400 nm) (Caimi and Eisenstark, 1986). This suggests that the mechanisms for repair of damage caused by NUV radiation are different from those for FUV radiation in *D. radiodurans*. It has also been reported that cells, when pretreated with a small, sublethal dose of NUV, become
resistant to a subsequent lethal dose of NUV (Caimi and Eisenstark, 1986) indicating the presence of an inducible defense system against NUV damage. Since NUV radiation produces hydrogen peroxide in the cell (McCormick et al., 1976), the major effects of NUV may be oxidative in nature (Caimi and Eisenstark, 1986). It is, therefore, possible that *D. radiodurans* may possess an inducible defense system against the lethal effects of hydrogen peroxide that can be induced by pretreatment with a sublethal dose of hydrogen peroxide. One of the goals of this work is to test this hypothesis.
CHAPTER 2. MATERIALS AND METHODS

Chemicals and enzymes

Hydrogen peroxide was purchased as 30% solution from Fisher Scientific (Nepean, Canada). Bovine liver catalase, diaminobenzidine and chloramphenicol were obtained from Sigma, horseradish peroxidase from ICN and restriction endonucleases from BRL.

Bacteria and plasmids

All Deinococcus species used in this study, Deinococcus radiodurans wild type R1(UWO288) and rec30 mutant, Deinococcus radiodurans Sark (UWO298), Deinococcus radiophilus (UWO1055), Deinococcus radiopugnans (UWO293) and Deinococcus proteolyticus (UWO1056) were kind gifts from Dr. R.G.E. Murray, University of Western Ontario, Canada. The plasmids used in this study were pAMkatE22 (katE), pBT22 (katG) and pDT1.5 (sodA).
Growth conditions

Deinococcus sp. and Escherichia coli K12 were grown at 30°C with shaking (200 rpm) in TGYM medium, which consists of 5 g of tryptone, 3 g of yeast extract, 1 g of glucose and 1 g of methionine per liter (pH 7.0). The other E. coli stains containing recombinant plasmids were grown at 37°C in LB medium, which consists of 10 g tryptone, 5 g yeast extract and 10 g sodium chloride per liter (pH 7.0). The growth of bacteria was measured by determining optical density (OD) at 600 nm.

Effects of Mn(II) and Mg(II) on growth and catalase activity of

D. radiodurans

Exponentially-growing cultures of D. radiodurans were inoculated into TGYM medium to an initial OD$_{600}$ of 0.02 and MnCl$_2$ or MgSO$_4$ were added to the cultures to a varying concentration and incubated at 30°C with shaking (200 rpm). The samples were removed periodically and growth was measured by determining OD$_{600}$ and catalase activity was determined as described as below.
Response to chemical oxidants

The effect of chemical oxidants was measured using a disc inhibition assay as described previously by Schellhorn and Hassan (1988). Late log-phase cultures of *Deinococcus* species and *E. coli* were poured onto TGYM agar plates and kept at room temperature for about 5 minutes and then the excess liquid was removed from the plates. After the plates had dried, filter paper discs (7 mm) were placed on the plates and the indicated amounts of H$_2$O$_2$ and paraquat were added onto the disc in 10 µl aliquots. The growth was measured and recorded as diameter of the zone of inhibition (including the 7-mm diameter of the filter disc) after 24 h and two day incubation at 30°C for *E. coli* and *Deinococcus* sp., respectively.

Preparation of cell extracts

Cells were harvested by centrifugation at 10,000 x g for 10 minutes, resuspended in 50 mM phosphate buffer (pH 7.0) containing 0.1 mM MgSO$_4$ and 150 µg/ml chloramphenicol (to prevent further biosynthesis) and washed three times with the same buffer. The pellet was resuspended in the buffer to an OD$_{600}$ of approximately 10. Cell suspensions were sonicated at 4°C (30 s pulse on, 20 s pulse off) for a total of 20 minutes using a Heat Systems Model XL2020 sonicator (Heat Systems Ultrasonics, Farmingdale, New York). Cell debris was removed by centrifugation (12,000 x g, 10
min, 4°C) in a microfuge and the cell extracts were assayed for catalase activity immediately after preparation or stored on ice until assay.

**Determination of protein**

Protein concentration in cell extracts was measured using the Bradford (1976) protein dye assay (Bio-Rad Laboratories, Mississauga, Canada) with bovine gamma globulin as the protein standard. Briefly, 0.8 ml of standards and appropriately diluted samples was added to small tubes and 0.2 ml of the reagent was added, mixed. After a period of 20 minutes, the absorbance was determined at 595 nm. OD$_{595}$ was plotted with the concentration of standards and the concentration of the unknown samples was determined with reference to a standard curve.

**Enzyme activity assay**

Catalase activity in whole cells and in cell extracts was determined by measuring the rate of hydrogen peroxide decomposition at 240 nm (Beers and Sizer, 1952). Hydrogen peroxide substrate was prepared by adding 0.425 ml of 30% H$_2$O$_2$ into 100 ml of 50 mM phosphate buffer (pH 7.0), yielding a stock solution containing 36.5 mM H$_2$O$_2$. Appropriate amount of samples was placed in cuvette and the phosphate buffer was added to 0.5 ml. Reaction was started by adding 0.5 ml of the H$_2$O$_2$ substrate. The
changes in $\text{OD}_{240}$ were followed for 4 minutes. One unit was defined as $1 \, \mu\text{mol}$ of hydrogen peroxide hydrolysed per minute.

**Polyacrylamide gel electrophoresis (PAGE)**

Non-denaturing polyacrylamide gels (7.5% and 10% for catalase and SOD activity staining, respectively) were prepared using Bio-Rad minislab gel system (Schellhorn and Stones, 1992). Samples of cell extracts were separated electrophoretically on the gels at a constant current of 30 mA in an ice water bath. Gels were stained for catalase activity using the peroxidase-diaminobenzidine method (Clare et al., 1984). In brief, the gels were soaked for 30 minutes in 50 mM phosphate buffer (pH 7.0) containing horseradish peroxidase (1.5 mg/30 ml) and for 10 minutes in $\text{H}_2\text{O}_2$ solution (4.7 mM). The gels were then rinsed twice with water and soaked in diaminobenzidine solution (15 mg/30 ml) with shaking until bands appeared. The nitroblue tetrazolium method was used for SOD staining (Beauchamp and Fridovich, 1971).

**Culture methods for killing experiments**

*D. radiodurans* R1 and *E. coli* were grown in TGYM medium at 30°C with shaking (200 rpm). Exponentially-growing cells were subcultured into the same medium to an initial $\text{OD}_{600}$ of 0.1. When cells reached an $\text{OD}_{600}$ of 0.2, 30% $\text{H}_2\text{O}_2$ was added...
to varying final concentrations and the samples incubated for 60 minutes. The cells were then serially diluted with sterile 50 mM phosphate buffer (pH 7.0) and plated immediately in triplicate on TGYM agar plates. Colonies were counted after two day growth at 30°C. For pretreatment experiments, exponentially-growing \textit{D. radiodurans} R1 cultures (initial OD$_{600}$ of 0.2) were treated with 5 mM H$_2$O$_2$ (final concentration) in the presence or absence of 100 \textmu g/ml chloramphenicol. After 60 minute incubation, 80 mM H$_2$O$_2$ was added to all treated cultures. Viability was assessed as described above. For UV killing experiments, after H$_2$O$_2$ pretreatment (in a final concentration of 10 mM) for 60 minutes, the cells were washed three times with 50 mM phosphate buffer (pH 7.0) and resuspended in the same buffer. Cell suspensions were UV irradiated in open Petri dishes under a germicidal lamp at a dose rate of 200 J/m$^2$ per minute [calibrated using a J-225 shortwave UV meter (Ultraviolet products, San Gabriel, CA.)]. At the indicated times, 0.1 ml of samples was removed, diluted with 50 mM phosphate buffer (pH 7.0) and plated on TGYM plates to assess viability. For \gamma-irradiation experiments, the washed cell suspensions were prepared in the same way as for UV killing except samples were separated into sterile microfuge tubes in aliquot (1 ml) and irradiated in parallel at a dose of 275 krad/h from a $^{60}$Co source. The samples were kept on ice during and after the irradiation. Survival of the samples were measured in the same way as that for after UV radiation.
**Effect of hydrogen peroxide on catalase expression**

Exponentially-growing cells of *D. radiodurans* R1 were inoculated into fresh TGYM medium to initial OD$_{600}$ of 0.1. When cells reached an OD$_{600}$ of 0.2, H$_2$O$_2$ was added to varying final concentrations as indicated. After 60 minute incubation, the cells were harvested by centrifugation (10,000 x g for 10 min.). Cell extracts were prepared by sonication (see above) and catalase assays were performed as described above. In cases where an increase in catalase activity was observed, samples were separated electrophoretically on non-denaturing PAGE to identify which catalase species was induced.

**Isolation of DNA**

Isolation of plasmid DNA was performed as described by Sambrook *et al.* (1989). Cells from 5 ml of overnight culture were centrifuged, and resuspended in 100 μl lysis buffer [25 mM Tris (pH 8.0), 10 mM EDTA (pH 8.0), 50 mM glucose] and incubated at room temperature for 5 min. A 200 μl volume of freshly prepared solution composed of 0.2 M NaOH and 1% (wt/vol) SDS was added, and the suspension incubated on ice for 5 minutes. A 150 μl volume of cold 3 M sodium acetate (pH 4.8) was added and the incubation continued for at least 15 minutes. After centrifugation at 13,000 rpm (microfuge) for 10 min, the supernatant was recovered and extracted once with an equal
volume of phenol, once with equal volume of phenol-chloroform (1:1) and once with equal volume of chloroform. The DNA was precipitated with 2 volumes of 95% ethanol, washed with 70% ethanol and dried by vacuum evaporation. The DNA was dissolved in TE buffer. The plasmid DNAs were purified by equilibrium centrifugation in CsCl-ethidium bromide gradients (Sambrook et al., 1989). Chromosomal DNA of Deinococcus sp. was isolated as described previously (Smith et al., 1988).

Southern blotting analysis

Chromosomal DNA from D. radiodurans R1 and Sark, D. radiophilus, D. proteolyticus, D. radiopugnans and E. coli was completely digested with restriction endonuclease and the fragments were separated by electrophoresis on agarose gel. The gel was soaked in a solution of 0.2 M NaOH and 0.4 M NaCl for 30 minutes with occasional rocking, and then washed in four changes of 0.1 X TAE buffer (4 mM Tris, 2 mM sodium acetate, 0.1 mM EDTA, pH 7.4) for 30 minutes. The DNA was transferred to a nylon membrane (Bio-Rad Laboratories, Richmond, CA) in pre-chilled 0.1 X TAE at 4°C, 200 mA for 1h. The membrane was placed on a pad of filter paper saturated with 0.4 M NaOH for 5 minutes and then washed in 2X SSC (300 mM NaCl, 30 mM trisodium citrate). The DNA was fixed to the membrane by baking at 80°C for 30 minutes. The plasmid DNA was labelled by using random primed DNA labelling (nonradioactive kit). Hybridization was performed for 24 h at 42°C in the presence of
hybridization buffer [50% formamide, 0.25 M sodium phosphate buffer (pH 7.2), 0.2 M NaCl, 7% (wt/vol) SDS, 1 mM EDTA (pH 8.0)]. The membranes were washed in the following solutions at room temperature with agitation for 20 minutes each: 1) 2 X SSC and 0.1% SDS, 2) 0.5 X SSC and 0.1% SDS and 3) 0.1 X SSC and 0.1% SDS. The hybridizing bands were detected by enzyme immunoassay as described by the manufacturer.
CHAPTER 3. RESULTS

Effects of Mn(II) and Mg(II) on growth and catalase activity

of Deinococcus radiodurans R1

An appropriate chemically-defined medium for Deinococcus sp. has not been described (Little et al., 1973; Shapiro et al., 1977) and the requirements for the optimal growth and expression of high radiation resistance of Deinococcus sp. are largely unknown. Manganese has been suggested to be important in radiation resistance, since Deinococcus radiodurans accumulates manganese in large amounts (Leibowitz et al., 1976) and is required for the activity of a DNA repair enzyme (Evans and Moseley, 1985). Recently, Chou and Tan (1990) have reported that manganese, at 2.5 μM or higher (0.3 mM), induces cell division and increases catalase and SOD activities in aging cultures of D. radiodurans IR. However, manganese also increases sensitivity to lethality mediated by both UV and ionizing radiation (Chou and Tan, 1990). In this study, we found that the Mn(II) stimulated the growth of D. radiodurans at a concentration of 0.2 mM or lower when cultures entered late exponential/early stationary phase (Fig. 1A), this is consistent with the results of Chou and Tan (1990). We also found that manganese inhibited growth at concentrations of 0.4 mM or higher which is
not reported in literature (Fig. 1A). The Mn(II) inhibited catalase activity of *D. radiodurans* at all the tested concentrations (Fig. 1B), this does not agree with Chou and Tan (1990). The Mg(II) stimulated growth (Fig. 2A) but appeared to have no effect on the catalase activity of *D. radiodurans* (Fig. 2B).

**Antioxidant enzymes produced by *Deinococcus* sp.**

To better understand how the *Deinococcus* sp. respond to oxidative stress, the antioxidant enzymes produced by these bacteria were measured. Relatively high catalase activities were found in the five tested strains of four *Deinococcus* species compared to that in *E. coli* and stationary phase cells had higher catalase activity than exponential phase cells in all tested strains (Table 1). These results were consistent with those reported for *D. radiodurans* IR (Chou and Tan, 1990). The types of catalases and superoxide dismutase produced by *Deinococcus* sp. were identified by visualization of the enzyme activities on non-denaturing polyacrylamide gels. *D. radiopugnans* produced only one catalase band, while the other species had two catalases, but with different electrophoretic mobility from catalases from *E. coli* (Fig. 3). All of *Deinococcus* sp. produced one SOD band except *D. radiophilus* which produced two SOD bands (Fig. 4). *D. radiodurans* IR has two catalases and three SOD (Chou and Tan, 1990).
Fig. 1. Effects of manganese on growth (A) and catalase activity (B) of \textit{D. radiodurans} R1. Exponentially-growing cultures were inoculated into an initial \textit{OD}_{600} of 0.02 and manganese was added to the cultures to various concentrations and incubated at 30°C with shaking (200 rpm). The samples were removed at times indicated and growth and catalase activity were measured as described in Materials and Methods.
Fig. 2. Effects of magnesium on growth (A) and catalase activity (B) of *D. radiodurans* R1. Exponentially-growing cultures were inoculated into an initial OD<sub>600</sub> of 0.02 and magnesium was added to the cultures to various concentrations and incubated at 30°C with shaking (200 rpm). The samples were removed at times indicated and growth and catalase activity were measured as described in Materials and Methods.
A

B

- Control
- 0.1 mM
- 1 mM
Table 1. Catalase Activity in *Deinococcus* sp.

<table>
<thead>
<tr>
<th>Species or Strain</th>
<th>Catalase Activity (U/mg of Protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Exponential</strong></td>
</tr>
<tr>
<td><em>D. radiodurans</em> R1 (UWO288)</td>
<td>548 ± 8</td>
</tr>
<tr>
<td><em>D. radiodurans</em> Sark (UWO298)</td>
<td>584 ± 15</td>
</tr>
<tr>
<td><em>D. radiophilus</em> (UWO1055)</td>
<td>511 ± 10</td>
</tr>
<tr>
<td><em>D. proteolyticus</em> (UWO1056)</td>
<td>620 ± 23</td>
</tr>
<tr>
<td><em>D. radiopugnans</em> (UWO293)</td>
<td>265 ± 16</td>
</tr>
<tr>
<td><em>E. coli</em> K12</td>
<td>4.3 ± 0.3</td>
</tr>
</tbody>
</table>

The values are mean of three experiments ± standard error.
Fig. 3. Electropherogram showing catalase isozymes of *Deinococcus* sp. Each lane was loaded with 10 μg protein except for *E. coli* which was loaded with 100 μg protein. 7.5% non-denaturing PAGE gel was run at a constant current of 30 mA and stained for catalase activity as described in Materials and Methods. Lanes: 1. *D. radiodurans* R1, 2. *D. radiodurans* Sark, 3. *D. radiophilus*, 4. *D. proteolyticus*, 5. *D. radiopugnans*, 6. *E. coli* K12.
Fig. 4. Electropherogram showing SOD isozymes of *Deinococcus* sp. Each lane was loaded with 10 µg protein except for *E. coli* which was loaded with 100 µg protein. 10% non-denaturing PAGE gel was run at a constant current of 30 mA and stained for SOD activity as described in Materials and Methods. Lanes: 1. *E. coli* K12, 2. *D. radiodurans* R1, 3. *D. radiodurans* Sark, 4. *D. radiophilus*, 5. *D. proteolyticus*, 6. *D. radiopugnans*. 
Response to hydrogen peroxide and paraquat

As the first step to investigate the response of *Deinococcus* sp. to oxidative stress, the sensitivity of *Deinococcus* sp. to hydrogen peroxide and paraquat was tested by using a disc inhibition assay. All *Deinococcus* species except *D. radiophilus* were more resistant to hydrogen peroxide and more sensitive to paraquat inhibition than *E. coli*. *D. radiophilus* was resistant to both hydrogen peroxide and paraquat killing (Table 2). The growth inhibition by hydrogen peroxide somehow was not completely consistent with the catalase activity results (Table 1). To further confirm the resistance of *Deinococcus* sp. to hydrogen peroxide, *Deinococcus radiodurans* R1 was chosen to test the lethality mediated by hydrogen peroxide. When exponentially-growing cells of *D. radiodurans* R1 and *E. coli* were exposed to various concentrations of hydrogen peroxide for 60 minutes, *D. radiodurans* R1 exhibited much greater resistance to the lethal effect of hydrogen peroxide than did *E. coli*, showing a large shoulder in the survival curve (Fig. 5). *D. radiodurans* R1 survived in 40 mM H$_2$O$_2$ for 60 minutes with greater than 50% of viability (Fig. 5). Since *D. radiodurans* has high catalase activity, cells may quickly break down hydrogen peroxide as soon as hydrogen peroxide was added, resulting in the high resistance to killing by hydrogen peroxide. To test if the killing by hydrogen peroxide is a function of time, the exponentially-growing cells of *E. coli* and *D. radiodurans* R1 were exposed to 5 mM and 60 mM hydrogen peroxide, respectively, for different lengths of time. As shown in Fig. 6, the killing of *D. radiodurans* by
hydrogen peroxide was the function of time. \textit{D. radiodurans} and \textit{E. coli} exhibited similar killing patterns by hydrogen peroxide (Fig. 6).

**Expression of catalase in \textit{D. radiodurans} R1**

To correlate hydrogen peroxide resistance with high levels of catalase, studies of regulation of catalase in \textit{Deinococcus radiodurans} R1 and the response of \textit{D. radiodurans} R1 to hydrogen peroxide were initiated. The growth kinetics and catalase expression of \textit{D. radiodurans} in rich medium were determined (Fig. 7). Catalase levels in \textit{D. radiodurans} R1 increased during growth into stationary phase. To determine if one of the two species of the catalases produced by \textit{D. radiodurans} is specifically induced during late exponential/early stationary phase, cell extracts were separated on non-denaturing gels and stained for catalase activity. Two catalase bands were produced in \textit{D. radiodurans} R1. The upper band (catalase A) was clearly induced when the culture entered stationary phase. The lower band (catalase B) seemed to be slightly induced during growth into the stationary phase (Fig. 7). Similar results have been reported for \textit{E. coli} (Loewen et al., 1985a).
Table 2. Sensitivity of *Deinococcus* sp. to oxidants

<table>
<thead>
<tr>
<th>Oxidant</th>
<th>Amount (μg)</th>
<th>Zone of Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Deinococcus Strainsb</td>
</tr>
<tr>
<td></td>
<td></td>
<td>288 298 1055 1056 293</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>48</td>
<td>ni^c</td>
</tr>
<tr>
<td></td>
<td>96</td>
<td>9 ni ni ni 9 12</td>
</tr>
<tr>
<td></td>
<td>192</td>
<td>11 ni ni ni 12 15</td>
</tr>
<tr>
<td></td>
<td>384</td>
<td>16 8 8 9 16 17</td>
</tr>
<tr>
<td></td>
<td>768</td>
<td>21 9 10 13 20 22</td>
</tr>
<tr>
<td>Paraquat</td>
<td>1.5</td>
<td>28 18 ni 18 ni ni</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>30 27 ni 22 11 ni</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>34 31 ni 30 20 8</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>39 37 9 36 29 12</td>
</tr>
</tbody>
</table>

a. The zone of inhibition includes 7 mm diameter paper disc.


c. ni, no inhibition.
Fig. 5. Survival of *D. radiodurans* and *E. coli* after challenge with H$_2$O$_2$. *D. radiodurans* R1 and *E. coli* were grown in TGYM medium at 30°C with shaking (200 rpm). The exponentially-growing cells were inoculated into the same medium to an initial OD$_{600}$ of 0.1. When the OD$_{600}$ of the cells reached 0.2, 30% H$_2$O$_2$ was added to the cells to various concentrations and exposed for 60 minutes. The cells were then diluted with 50 mM phosphate buffer (pH 7.0) and plated on TGYM plates in triplicate to assess viability.
Surviving Fraction (%) vs. Hydrogen peroxide (mM)

- E. coli K12
- D. radiodurans R1
Fig. 6. Kinetics of killing of *D. radiodurans* and *E. coli* by H$_2$O$_2$. The exponentially-growing cells of *D. radiodurans* R1 and *E. coli* were inoculated into the TGYM medium to an initial OD$_{600}$ of 0.1 and grown at 30°C with shaking (200 rpm). When the OD$_{600}$ of the cells reached 0.2, the cells of *D. radiodurans* R1 and *E. coli* were exposed to 60 mM and 5 mM of H$_2$O$_2$, respectively, for varying lengths of time. Samples (0.1 ml) of cells were removed at the times indicated, diluted with 50 mM phosphate buffer (pH 7.0), and plated on TGYM plates in triplicate to assess viability.
Surviving Fraction (%) vs. Time (min)

- E. coli K12
- D. radiodurans R1
Fig. 7. Growth and catalase expression of *D. radiodurans*. *D. radiodurans* R1 was grown in TGYM medium at 30°C with shaking (200 rpm). The exponentially-growing cells were subcultured into the same medium to an initial OD$_{600}$ of 0.020. The samples were removed at times indicated and growth and catalase activity were measured as described in Materials and Methods. Protein samples (10 μg) were loaded on each lane of the gel and stained for catalase isozymes. The gel shows the two isozymes that were produced at each point of sampling time.
Adaptation of *D. radiodurans* R1 to hydrogen peroxide

It has been reported that *E. coli* and *S. typhimurium* are capable of adaptation to hydrogen peroxide in a similar manner (Demple and Halbrook, 1983; Christman *et al.*, 1985). Like *E. coli*, *D. radiodurans* has inducible catalase activity. It is possible that *D. radiodurans* may respond to hydrogen peroxide in a way similar to that in *E. coli*. To test this hypothesis, we tested adaptation of *D. radiodurans* to hydrogen peroxide. Exponentially-growing cells of *D. radiodurans*, when pretreated with 5 mM hydrogen peroxide for 60 minutes, became relatively resistant to killing by 80 mM hydrogen peroxide compared with the cells that had not been pretreated with hydrogen peroxide (Fig. 8). The cells pretreated with 5 mM hydrogen peroxide in the presence of chloramphenicol did not acquire resistance to killing by hydrogen peroxide. These data indicated that *de novo* protein synthesis was required for adaptation of *D. radiodurans* to hydrogen peroxide-mediated lethality.
Fig. 8. Induced resistance to H$_2$O$_2$ in D. radiodurans. D. radiodurans R1 was grown in TGYM medium at 30°C with shaking (200 rpm). Exponentially-growing cells were inoculated into the same medium to an initial OD$_{600}$ of 0.1. When cells had reached an OD$_{600}$ of 0.2, the cells were treated with 5 mM of H$_2$O$_2$ in the presence and absence of 100 µg/ml of chloramphenicol. After 60 minutes, 80 mM of H$_2$O$_2$ was added to all cultures. Aliquots were removed periodically, diluted in 50 mM phosphate buffer (pH 7.0) and plated on TGYM plates in triplicate to assess viability.
Surviving Fraction (%) vs. Time (min)

- Pretreated
- Not pretreated
- Pretreated + Cm
- Cm only
Catalase induction by hydrogen peroxide

*E. coli* and *S. typhimurium* induce catalase activity upon exposure to a low level of hydrogen peroxide (Demple and Halbrook, 1983; Christman *et al.*, 1985.). The catalase activity in the *D. radiodurans* R1 cells treated with a low dose of hydrogen peroxide was measured to determine if *D. radiodurans* induced catalase activity upon exposure to hydrogen peroxide. Increased catalase activity was observed in *D. radiodurans* R1 cells treated with hydrogen peroxide (from 0.5 mM to 15 mM) when compared with untreated cells (1.2 to 2.4 fold) (Fig. 9). Since *D. radiodurans* possesses two catalases, cell extracts were separated on non-denaturing PAGE gels to determine if one of the catalases was affected. The catalase A (upper band) was not induced by hydrogen peroxide, but the catalase B (lower band) was (Fig. 10). Similarly, in *E. coli* the upper band (HPII) is not induced by hydrogen peroxide but the lower band (HPI) is (Loewen *et al.*, 1985a).
Fig. 9. Induction of catalase in *D. radiodurans* by H$_2$O$_2$. Exponentially-growing cultures (OD$_{600} = 0.2$) were treated with various concentrations of H$_2$O$_2$ for 60 minutes. The cells were harvested by centrifugation (10,000 x g, 10 min, 4°C), washed with 50 mM phosphate buffer (pH 7.0) three times and resuspended in the same buffer. Cell extracts were prepared as described in Materials and Methods and assayed for catalase and protein.
Fig. 10. Electropherogram showing $\text{H}_2\text{O}_2$-mediated increases in catalase activity. Lane: 1, 4, not treated, 2, 5, treated with 5 mM $\text{H}_2\text{O}_2$, 3, 6, treated with 10 mM $\text{H}_2\text{O}_2$. 1-3, loaded with 5 $\mu$g of protein, 4-6, loaded with 0.5 $\mu$g of protein.
Induced resistance to UV- and γ-ray-mediated lethality

As UV and γ-rays can exert their lethality by generating reactive oxygen species (Repine et al., 1981; Sammartano and Tuveson 1983; Eisentark, 1989), the effect of hydrogen peroxide pretreatment on the resistance to UV and γ-ray radiation was tested. Exponential-phase cultures of *D. radiodurans* treated with 10 mM hydrogen peroxide were more resistant to the lethal effects of UV radiation (Fig. 11) and γ-radiation (Fig. 12) than untreated control cultures. The data also indicated that *de novo* protein synthesis was required for resistance of *D. radiodurans* to the lethal effect of UV radiation. This suggests a correlation between resistance to the lethal effects of UV and γ radiation and antioxidant mechanisms.
Fig. 11. H$_2$O$_2$-induced resistance to UV radiation in *D. radiodurans*. *D. radiodurans* R1 was grown in TGYM medium at 30°C with shaking (200 rpm). Exponentially-growing cells were inoculated into the same medium to an initial OD$_{600}$ of 0.1. When the OD$_{600}$ of the cells reached 0.2, the cells were treated with 10 mM of H$_2$O$_2$ (final concentration) in the presence and absence of 100 µg/ml of chloramphenicol for 60 minutes. Samples were centrifuged (10,000 x g, 10 min, 4°C), washed and resuspended in 50 mM phosphate buffer (pH 7.0) to an OD$_{600}$ of approximate 0.3. Samples (5 ml) were irradiated in uncovered Petri dishes under UV at a dose rate of 200 J/M$^2$/min. At indicated intervals, 0.1 ml of samples were removed, diluted in 50 mM phosphate buffer (pH 7.0) and plated on TGYM plates in triplicate to assess viability.
Fig. 12. H₂O₂-induced resistance to γ radiation in *D. radiodurans*. *D. radiodurans* R1 was grown in TGYM medium at 30°C with shaking (200 rpm). The exponentially-growing cells were inoculated into the same medium to an initial OD₆₀₀ of 0.1. When the OD₆₀₀ of the cells reached 0.2, 30% H₂O₂ was added to the cells to a final concentration of 10 mM and incubation continued for 60 minutes. The cells were then centrifuged (10,000 x g, 10 min, 4°C.), washed and resuspended in 50 mM phosphate buffer (pH 7.0) to an OD₆₀₀ of approximate 0.3. The cell suspensions were exposed to γ radiation at a dose rate of 275 krad/h and were kept on ice during radiation. Samples were removed, diluted in 50 mM phosphate buffer (pH 7.0) and plated on TGYM plates in triplicate to assess viability.
Sensitivity of cultures at different growth stages to UV radiation

Since cells at different growth stages have different levels of catalase activity, sensitivity of cultures at different growth stages to UV radiation was tested to determine if a correlation between the level of catalase and UV resistance can be established. Though the stationary-phase cells have a higher level of catalase activity, they did not show higher resistance to UV-mediated lethality than exponential-phase cells (Fig. 13). A correlation between high radiation resistance and high catalase activity can not be suggested by these results. The catalase-deficient mutants of *Deinococcus* sp. are needed to further confirm this.
Fig. 13. Sensitivity of cultures at different growth stages to UV radiation. Exponential-phase cultures of *D. radiodurans* R1 were inoculated into TGYM medium to an initial OD$_{600}$ of 0.02 and grown at 30°C with shaking. Samples removed at times indicated were centrifuged at 10,000 x g for 10 minutes, washed with 50 mM phosphate buffer (pH 7.0) and resuspended in the same buffer to an OD$_{600}$ of approximate 0.3. 5 ml of the cell suspension was irradiated in uncovered Petri dishes under UV at a dose of 500 J/M$^2$ and 0.1 ml of samples was removed, diluted with 50 mM phosphate buffer (pH 7.0) and plated on TGYM plates in triplicate to assess the viability.
Catalase and RecA in the defense of *D. radiodurans*

against H$_2$O$_2$-mediated lethality

The mechanisms of *E. coli* against oxidative stress have been extensively studied (Keele *et al.*, 1970; Yost and Fridovich, 1973; Yoshpe-Purer *et al.*, 1977; Dougherty *et al.*, 1978; Claiborne *et al.*, 1979; Imlay and Linn, 1986, 1987, 1988.). The conclusions regarding the relative importance of catalase and RecA in the protection of *E. coli* from lethal effects of hydrogen peroxide are inconsistent (Carlsson and Carpenter, 1980; Loewen, 1984.). To determine that the protection of *D. radiodurans* against hydrogen peroxide is a function of catalase and RecA, the response of a recA mutant of *D. radiodurans*, rec30 strain, to hydrogen peroxide was tested.

To confirm the phenotype of rec30 mutant, we examined the sensitivity of *D. radiodurans* rec30 mutant to UV radiation compared to the wild type strain. As shown in Fig. 14, the rec30 was more sensitive to UV radiation than the wild type and the sensitivity was comparable to that described by Moseley and Copland (1975).
Fig. 14. Sensitivity of *D. radiodurans* wild type and rec30 mutant to UV radiation. *D. radiodurans* R1 and rec30 mutant were grown in TGYM medium at 30°C with shaking. The exponentially-growing cells were inoculated into the same medium to an initial OD$_{600}$ of 0.1. When the OD$_{600}$ of the cells reached 0.2, the cells were centrifuged, washed and resuspended in 50 mM phosphate buffer (pH 7.0) to an OD$_{600}$ of approximate 0.3. 5 ml of the cell suspension was irradiated in uncovered Petri dishes under UV at a dose rate of 200 J/M$^2$/min. At intervals, 0.1 ml of samples was removed, diluted in 50 mM phosphate buffer (pH 7.0) and plated on TGYM plates in triplicate to assess viability.
100
10
0

(.

0.1
0.01
0.001

0 200 400 600 800 1000

UV dose (J/m²)

Survival fraction (%)
Response of *D. radiodurans* rec30 to hydrogen peroxide

When exponentially-growing cells of *D. radiodurans* wild type and rec30 mutant strains were exposed to hydrogen peroxide for 60 minutes at various concentrations, the rec30 mutant exhibited greater sensitivity to the lethal effects of hydrogen peroxide than did the wild type strain (Fig. 15). The shoulder in the survival curve of the rec30 mutant was smaller than that of the wild type strain (Fig. 15). These results suggest that RecA plays an important role in the defense against oxidative stress, as described in *E. coli* (Carlsson and Carpenter, 1980) and other bacteria (Hassett *et al.*, 1990).

Hydrogen peroxide pretreatment and protection of *D. radiodurans* rec30 against \( \text{H}_2\text{O}_2 \)- and UV-mediated lethality.

Catalase induction levels in the rec30 mutant were measured. Little difference between this mutant and the wild type was found (data not shown). As in the wild type, catalase was induced by hydrogen peroxide in the rec30 mutant (Fig. 16). To test the presence of adaptive response of this mutant to hydrogen peroxide, the rec30 mutant was pretreated with 1 mM of hydrogen peroxide for 60 minutes, and exposed to 30 mM hydrogen peroxide and UV light. We found that the hydrogen peroxide pretreatment did not result in any protection against lethal effects of hydrogen peroxide (Fig. 17) and UV radiation (Fig. 18).
Fig. 15. Inactivation of *D. radiodurans* wild type (R1) and rec30 mutant by H₂O₂. Exponentially-growing cells of *D. radiodurans* R1 and rec30 were inoculated into TGYM medium to an initial OD₆₀₀ of 0.1 and were incubated at 30°C with shaking (200 rpm). When the cells reached an OD₆₀₀ of 0.2, 30% H₂O₂ was added to the cells to various concentrations and exposed for 60 minutes. The cells were diluted with 50 mM phosphate buffer (pH 7.0) and plated on TGYM plates in triplicate to assess viability.
Fig. 16. Induction of catalase in *D. radiodurans* rec30 by \(\text{H}_2\text{O}_2\). The same procedures as those for *D. radiodurans* R1 were used.
Fig. 17. H$_2$O$_2$-induced resistance to H$_2$O$_2$ in *D. radiodurans* rec30 (A) compared with *D. radiodurans* R1 (B). Same protocols were used as described in Fig. 8 legend except that 1 mM of H$_2$O$_2$ was used for the pretreatment.
Fig. 18. H$_2$O$_2$-induced resistance to UV radiation in *D. radiodurans* rec30 (A) compared to *D. radiodurans* R1 (B). Same protocols were used as those described in Fig.11 legend, except that 1 mM of H$_2$O$_2$ was used for the pretreatment.
Homology between *E. coli* catalase and SOD genes

and those of *Deinococcus* sp.

As described in previous sections, *E. coli* and *D. radiodurans* exhibit similar adaptive response pattern to hydrogen peroxide. Furthermore, the catalase genes of *D. radiodurans* and *E. coli* are regulated in a similar way. Therefore, *E. coli* catalase genes can potentially serve as probes to clone the catalase genes from *Deinococcus* sp. since they have been successfully used as probes to identify and clone similar genes in other bacteria (Switala et al., 1990). Homology between *E. coli* catalase and SOD genes and those of *Deinococcus* sp. was measured by hybridization of DNA from *Deinococcus* species with plasmids which contain *E. coli* katG, katE and sodA genes. Results (Fig. 19) showed that there was no homology between DNA sequence of *Deinococcus* sp. and the katE gene of *E. coli*, when the plasmid pAMkate22 which contains the *E. coli* katE gene was used as the probe. Similar results were obtained when the plasmids, pBT22 (katG) and pDT1.5 (sodA) were used as probes.
CHAPTER 4. DISCUSSION

The radiation resistant bacterial genus, *Deinococcus*, formerly a part of *Micrococcus* (Murray, 1981), consists of four species which share extremely high resistance to both the lethal and mutagenic effects of ionizing and ultraviolet (UV) radiation (Moseley, 1983). The high radiation resistance of the type species of *Deinococcus*, *D. radiodurans*, has been attributed to an effective constitutive DNA repair system (Moseley and Laser, 1956; Moseley *et al*., 1972; Moseley and Copland, 1975; Evans and Moseley, 1983; Moseley and Evans, 1983). Only two mechanisms for repair of UV-damaged DNA have been found in *D. radiodurans* (Boling and Setlow, 1956; Moseley *et al*., 1972; and Moseley and Copland, 1975). There does not appear to be an adaptive DNA repair system analogous to that of the *E. coli* SOS system in the *Deinococcus* sp.. Antioxidant mechanisms may also be involved in the radiation resistance, since ionizing and near UV radiation can exert their lethal effects by generation of active oxygen species (McCormick *et al*., 1976; Repine *et al*., 1981; Eisenstark, 1989). However, the mechanisms involved in the protective response of these bacteria to oxidative stress are poorly understood. DNA repair systems and protective enzymes including catalase, superoxide dismutase (SOD) and peroxidase are generally considered to play an important role in the defense against oxidative stress.
(Fridovich, 1978; Imlay and Linn, 1986, 1987, 1988; Sancar, 1988; Walker, 1984). The purpose of this study was to identify the antioxidant enzymes produced in *Deinococcus* sp., to test the antioxidant response of these bacteria to oxidative stress, and to determine if a correlation between radiation resistance and antioxidant enzymes can be established.

**Antioxidant enzymes (catalase and SOD) and normal aerobic life**

Relatively high levels of catalase and SOD in *D. radiodurans* IR have been reported (Chou and Tan, 1990). Two catalases and three SODs are produced in *D. radiodurans* IR. In this study, high levels of catalase and SOD have been found in other five strains of *Deinococcus* sp. as well. All the tested strains possess two catalases except *D. radiopugnans* which has one catalase. The reason that *Deinococcus* sp. have high catalase and SOD activities has not been determined, but there are several possible explanations. *Deinococcus* sp. are aerobic and the aerobic life-style offers the cells an energetic advantage. However, utilization of oxygen results in a deleterious consequences, since partially reduced oxygen intermediates are toxic and mutagenic to cells (McCormick et al., 1976; Fridovich, 1978; Moody and Hassan, 1982). While evolving mechanisms for the utilization of oxygen, aerobic organisms had to develop defense mechanisms to cope with its toxicity. To face the threat from the production of active oxygen species during aerobic metabolism, *Deinococcus* sp., like other aerobic microorganisms, may mobilize considerable resources for eliminating the reactive oxygen
species and repairing the oxidative damage. Catalase and superoxide dismutase are examples of such evolved defense mechanisms.

The natural habitat of the *Deinococcus* sp. is uncertain, but most likely it is the soil environment (Murray, 1986). Radiation-resistance has been used as a selective factor for isolation, but it can not be argued that radiation is a selective agent during the evolution of *Deinococcus* sp., since radiation-resistant strains have also been isolated without prior exposure to radiation (Murray and Robinow, 1958; Christensen and Kristensen, 1981). These bacteria may live together with other organisms since, for good growth, they require amino acids such as methionine and some strains seem to require vitamins or yeast extract (Raj *et al*., 1960; Shapiro *et al*., 1977; Ito *et al*., 1983). They are probably poor competitors since they are relatively slowly growing bacteria. Thus they are not easily isolated without prior irradiation.

**The response of *Deinococcus* sp. to oxidative stress**

Although the data from the disc inhibition assay does not unequivocally show that the more catalase the strain has, the more resistant to hydrogen peroxide it is, it is clear that *Deinococcus* species are more resistant to hydrogen peroxide than *E. coli*. The further study on the sensitivity of *D. radiodurans* R1 to hydrogen peroxide added to broth cultures supports the idea that *Deinococcus* sp. are highly resistant to the lethal effects of hydrogen peroxide. The high resistance of *Deinococcus* sp. to the lethal effects
of hydrogen peroxide may reflect the fact that *Deinococcus* sp. have high levels of
catalase and an effective constitutive DNA repair system. However, they show
sensitivity to paraquat. Paraquat, widely used as a herbicide, is thought to undergo
oxidation-reduction cycling within cells thereby generating intracellular O$_2^-$ in that it is
easily reduced to a stable radical, which then reacts with oxygen, generating superoxide
radicals. It has been reported that strains which overproduce superoxide dismutase are
more sensitive to paraquat than strains with normal levels of this enzyme. This suggests
that overproduction of SOD may result in more dismutation of superoxide, generating
excess H$_2$O$_2$ and OH$,\,$ which are relatively harmful to cells (Bloch and Ausubel, 1986;
Scott *et al.*, 1987). This may also be the case in *Deinococcus* sp.. But why *D.*
radiophilus has high SOD activity and has resistance to both hydrogen peroxide and
paraquat is still an unresolved question.

The regulation of catalase in *Deinococcus* sp.

The chemical requirements for optimal growth and expression of antioxidant
enzymes are poorly understood. Chou and Tan (1990) have recently reported that
manganese induces cell division and increases catalase and SOD activities in aging
cultures of *D. radiodurans* IR. In this study, we have found that, consistent with
previous results reported for *D. radiodurans* IR (Chou and Tan, 1990), manganese
stimulates cell growth of *D. radiodurans* R1 at low concentrations (0.2 mM or lower).
However, manganese inhibits cell growth at a concentration of 0.4 mM or higher and decreases catalase activity at all the concentrations tested. The difference may be that the effects of manganese on *Deinococcus* sp. are species specific. What manganese does in *Deinococcus* sp. is not clear. It has been reported that *D. radiodurans* can accumulate manganese in large amounts and that the manganese is associated with the chromosome (Leibowitz *et al.*, 1976). Manganese is known to interact preferentially with the guanine moiety in nucleoside, dinucleoside monophosphates and DNA (Anderson *et al.*, 1971) and alters the interaction between DNA and proteins (Eisinger *et al.*, 1965). Therefore, it is possible that manganese may serve as a co-effector in the expression of some regulons associated with starvation.

*D. radiodurans* R1, the type species of genus *Deinococcus*, produces two catalases (catalase A and B) on non-denaturing PAGE and the two catalases are regulated independently. Catalase A (upper band) is induced during growth into stationary phase, whereas catalase B (lower band) is only induced slightly if at all when cells enter stationary phase (Fig. 7). In addition, catalase A is not induced by hydrogen peroxide, but catalase B is (Fig. 10). These findings suggest that the systems controlling the synthesis of the two catalases may respond to different stimuli and may involve different mechanisms.

Similar results are also found in *E. coli*. *E. coli* produces two catalases (HPI and HPII). HPI (lower band) is not induced during growth into stationary phase but HPII (upper band) is; HPI is induced by hydrogen peroxide but HPII is not (Loewen *et al.*,...
1985). HPI (encoded by the *katG* gene) synthesis is under the control of the *oxyR* regulon which responds to oxidative stress (Christman *et al.*, 1985; Morgan *et al.*, 1986). In contrast, HPII (encoded by *katE* gene) synthesis is induced by oxygenation and by the onset of the stationary phase (Loewen *et al.*, 1985a) and may be affected by intracellular pH (Schellhorn and Hassan, 1988). The synthesis of HPII is controlled by the *katF* regulon.

As discussed above, catalase A resembles HPII, and catalase B is similar to HPI in regulation and response to hydrogen peroxide. We do not yet know whether or not *D. radiodurans* possesses an *oxyR*-like regulon or a *katF*-like regulon but these results at least provide information for comparison of catalase synthesis in different bacterial species and for a better understanding of the regulation of catalase in these bacteria.

**Adaptive response of *D. radiodurans* to hydrogen peroxide**

Exposure of *E. coli* and *S. typhimurium* to a sublethal level of hydrogen peroxide induces a protective response which confers increased resistance to subsequent lethal exposure (Demple and Halbrook, 1983; Christman *et al.*, 1985). Induced in this response, are at least two protective regulons: *oxyR* and SOS. The former encodes a positive effector and activates the synthesis of scavenging enzymes including catalase, alkylhydroperoxide reductase, and other proteins (Christman *et al.*, 1985), which thereby can minimize damage by an enhanced scavenging of active oxygen species. The SOS
system enhances recombinational DNA repair. In this study, we found that, like *E. coli* and *S. typhimurium*, *D. radiodurans* shows increased resistance to lethal levels of hydrogen peroxide when pretreated with a sublethal level of hydrogen peroxide (Fig. 8.). The induced proteins have not been identified but catalase is found to be induced (Fig. 9). These results suggest that *D. radiodurans* possesses inducible defense mechanisms against the deleterious effects of hydrogen peroxide. Since ionizing and NUV irradiation can exert lethality by producing reactive oxygen species (Repine *et al.* 1981; Eisenstark, 1989), the effects of H₂O₂ pretreatment on the resistance of *D. radiodurans* to UV and γ radiation were tested to further support the view. As expected, the pretreated cells were found to be relatively resistant to the lethal effects of UV and γ radiation. A correlation between UV and ionizing radiation resistance and antioxidant mechanisms can be suggested by this finding.

The hypothesis that *D. radiodurans* possesses inducible mechanisms against hydrogen peroxide can also be supported indirectly by the findings from Caimi and Eisenstark (1986). They have reported that although *Deinococcus* sp. are extremely resistant to the lethal effects of far-ultraviolet radiation (FUV, 254 nm), *D. radiodurans* is highly sensitive to killing by near-ultraviolet radiation (NUV, 300-400 nm), and cells become resistant to a subsequent lethal dose of NUV when pretreated with a small dose of NUV (Caimi and Eisenstark, 1986). This suggests that the mechanisms for repair of damage caused by NUV radiation are different from those for FUV radiation in *D. radiodurans*, and indicates the presence of an inducible defense system against NUV
damage in *D. radiodurans*. Since NUV radiation produces hydrogen peroxide in the cell (McCormick *et al.*, 1978), the major effects of NUV may be oxidative in nature (Caimi and Eisenstark, 1986).

Overlaps between oxidative stress and other stress responses have been observed in *E. coli* and *S. typhimurium*. When cells are pretreated with a low dose of hydrogen peroxide, they become cross resistant to heat shock and other types of stress (Christman *et al.*, 1985). Some of the proteins induced by hydrogen peroxide or superoxide, including some DNA repair enzymes, are also induced by heat shock, starvation, and other types of stress. The SOS response, which is induced following serious damage to a bacterial cell DNA, may be important for the defense against oxidative stress as well. Since *D. radiodurans* does not appear to have a SOS system and since the two repair mechanisms for UV damage found so far are constitutive (Moseley, 1983), a new response whose biochemical and genetic basis is unknown may be induced in response to hydrogen peroxide. Our finding that the rec30 mutant of *D. radiodurans* does not exhibit increased resistance to the lethal effects of hydrogen peroxide, when pretreated with a low concentration of hydrogen peroxide (Fig. 17a.) suggests that an analogue of RecA is involved in the inducible mechanism(s).
Possible roles of catalase and RecA in the defense of *D. radiodurans* against hydrogen peroxide

Killing of *E. coli* by hydrogen peroxide has been shown to proceed by two modes (Imlay and Linn, 1986). Mode-one killing, occurring at low concentrations of hydrogen peroxide (1 mM to 3 mM), appears to be due to DNA damage, since strains deficient in RecA protein, exonuclease III, RecBC, or polymerase I are hypersensitive to this mode of killing (Imlay and Linn, 1986). Mode-two killing occurs at higher concentrations of hydrogen peroxide (Imlay and Linn, 1986). The damage for mode-two killing has not been characterized. The resistance to mode-two killing in *E. coli* has been attributed to catalase activity (Imlay and Linn, 1987). RecA and catalase are thought to play an important role in the defense against hydrogen peroxide (Carlsson and Carpenter, 1980; Loewen, 1984; Hassett *et al.*, 1990). However, the conclusions regarding the importance of RecA and catalase in this defense are inconsistent. Carlsson and Carpenter (1980) emphasize the role of RecA in the defense of *E. coli* against hydrogen peroxide, while Loewen (1984) suggests that catalase is more important.

RecA protein is a positive regulator of the SOS system in response to blockage of the replication fork, it cleavages the LexA protein, a transcriptional repressor of the genes of the regulon. RecA protein is involved in recombinational events including those which repair DNA damage. Strains with mutations in *recA* gene are hypersensitive to hydrogen peroxide (Imlay and Linn, 1988). This sensitivity could be due to its inability
to induce SOS functions, its inability to carry out DNA recombination, or both.

In this study, the shape of the killing curve of *D. radiodurans* rec30 by hydrogen peroxide suggests that mode-one and mode-two killing can both occur in *D. radiodurans*. Since there is no SOS system in *Deinococcus* sp., the sensitivity of *D. radiodurans* to hydrogen peroxide is due to deficiency in recombinational repair and/or an unidentified system which requires *recA*. The bacteria may respond to oxidative stress by a combination of these mechanisms and DNA repair systems. Scavenging enzymes such as catalase and SOD may serve as a first line of defense against oxidative stress by preventing the accumulation of reactive oxygen species. As a second line of defense against oxidative stress, repair enzymes including RecA may protect cells by repairing the DNA damage caused by reactive oxygen species.

**Homology between *E. coli* catalase and SOD genes**

and those of *Deinococcus* sp.

*E. coli* produces two catalases (HPI and HPII) and two superoxide dismutases (MnSOD and FeSOD). The genes encoding these enzymes have been cloned (Sakamota and Touati, 1984; Touati, 1983; Triggs-Raine *et al.*, 1988; von Ossowski *et al.*, 1991). At least the catalase genes of *E. coli* have been shown to be useful for screening other bacteria (Switala *et al.*, 1990). The similarity of *D. radiodurans* and *E. coli* in catalase patterns, regulations and response to hydrogen peroxide stimulated us to try to identify
and clone catalase genes from *Deinococcus* sp. by using cloned *E. coli* catalase genes as a probe. Our attempts to identify *Deinococcus* sp. DNA sequences homologous to *E. coli* antioxidant enzyme genes (*katG, katE* and *sodA*) using Southern blot analysis were unsuccessful. These results and other findings (Switala *et al.*, 1990) support the view that there is significant divergence in antioxidant enzyme genes of bacteria.

**Conclusions**

1. High levels of catalase were found in all five tested strains of *Deinococcus* sp.
2. The types of catalase and SOD produced by *Deinococcus* sp. were identified. Two catalases and one SOD were produced in the strains of *Deinococcus* sp. except *D. radiopugnans* which produced one catalase, and *D. radiophilus* which produced two SODs.
3. These bacteria were found to be resistant to hydrogen peroxide relative to *E. coli* but sensitive to paraquat.
4. The two catalases of *D. radiodurans*, like those of *E. coli*, were found to be regulated independently.
5. Manganese was found to stimulate or inhibit the growth of *D. radiodurans* at low or high concentrations, respectively, and decrease catalase activity. Unlike manganese, magnesium increased the cell growth, but had no effect on catalase activity.
6. Resistance to the lethal effects of hydrogen peroxide and ionizing and UV radiation
can be induced by pretreatment with sublethal levels of hydrogen peroxide in *D. radiodurans*.

7. Resistance to lethal effects of hydrogen peroxide and UV radiation can not be induced by pretreatment with sublethal levels of hydrogen peroxide in a *D. radiodurans* rec30 mutant.

8. Homology between *E. coli* catalase and SOD genes and those of *Deinococcus* sp. was not found.
REFERENCES


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