MOLECULAR DIVERSITY AND HEAVY METAL INTERACTIONS IN *Deinococcus* spp.

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DECLARATION

STATEMENT UNDER O. Ph.D. 8/ (iii) OF

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The work presented in this thesis has been carried out by me under the guidance of Dr. G. Archana, Department of Microbiology and Biotechnology Centre, Faculty of Science, The M. S. University of Baroda, Vadodara, Gujarat, India. The data reported herein is original and has been derived from studies undertaken by me.

Place: Vadodara **Ruchi Chaturvedi**

Date: Candidate

This is to certify that the above declaration is true.

Place: Vadodara **Dr. G. Archana**

Date: Research Supervisor

Dedicated

to my family

Acknowledgement

"None of us got where we are solely by pulling ourselves up by our bootstraps. We got here because somebody - a parent, a teacher, an Ivy League crony or a few nuns - bent down and helped us pick up our boots. ~Thurgood Marshal"

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Ruchi Chaturvedi

Table of Contents

List of Abbreviations

2-DE	2 –dimensional gel electrophoresis
AAS	Atomic absorption spectrophotometer
Amp^r	Ampicillin resistance
ATCC	American type culture collection
BER	Base excision repair
BLAST	Basic local alignment search tool
bp	Base pair
Cat^r	Chloramphenicol resistance
cfu	Colony forming unit
CTAB	Cetyl trimethylammonium bromide
DGGE	Denaturing gradient gel electrophoresis
DR1	*Deinococcus radiodurans* R1
DSBs	Double stranded breaks
EDTA	Ethylene diamine tetra acetic acid
EMP	Embden–Meyerhof–Parnas pathway
H_2DCFDA	2′,7′-dichlorodihydrofluorescein diacetate
ICP-AES	Inductively coupled plasma- Atomic Emission spectroscopy
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl-1-thio-L-D-galactopyranoside
IR	Ionising radiation
kbp	Kilo-basepair
kDa	Kilo-dalton
kGy	Kilo Gray
mol	Mole
MCO	Metal catalysed oxidation
MT	Metallothionein

MTCC	Microbial type culture collection
NER	Nucleotide excision repair
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PC	Carbonylated protein
PCR	Polymerase chain reaction
PPP	Pentose phosphate Pathway
RE	Restriction Endonuclease
ROS	Reactive Oxygen Species
rRNA	Ribosomal ribonucleic acid
SDS	Sodium dodecyl sulphate
SOD	Superoxide Dismutase
TAE	Tris Acetate EDTA
T-BARs	Thio-barbituric acid reactive substances
TCA	Tricarboxylic acid
TE	Tris EDTA
Tris	Tris (hydroxymethyl) aminomethane
UV	Ultraviolet

Note: The full forms of several rarely used abbreviations have been described within the text

List of Symbols

α	Alpha
β	Beta
γ	Gamma
μ	Micro
k	Kilo
l	Litre
m	Milli (10^{-3})
n	Nano (10^{-9})
p	Pico (10^{-12})
%	Percentage
g	Gram
min	Minutes
h	Hours
s	Second
M	Molar
U	Unit
V	Voltage

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Chapter 1: Review of literature

There is something fascinating about science. One gets such wholesale returns of conjecture out of such a trifling investment of fact. ~Mark Twain

Introduction

The ability of certain bacterial cells to resist ionising radiation is unusual due to apparent absence of niches that expose life forms to ionising radiation on Earth. Radiation resistance bacteria are represented in both the eubacteria and archaea, among which *Deinococcus*, *Rubrobacter* and *Kineococcus* represent genera whose members are largely radiation resistant. Most of the radiation-resistant bacteria reported are gram positive, with the exception of a radiation resistant gram-negative cyanobacterium, *Chroococcidiopsis* (Billi et al., 2000), gram positive species such as *Micrococcus luteus* (*Sarcina lutea*). *Methylobacterium radiotolerans* (Green and Brousfield, 1983), *Lactobacillus plantarum* (Hastings et al., 1983), *Acinetobacter radioresistens* (Nushimura and Izuka, 1988), *Enterococcus faecium* (van Gerwen, 1999), *Hymenobacter actinosclerus* (Collins et al., 2000), *Kocuria rosea* (Brooks and Murray, 1981) are some radiation resistant gram positive bacteria. Radiation resistance is widespread among hyperthermophilic archaea, for example, *Pyrococcus furiosus* (DiRuggiero, 1997), *Thermococcus gammatolerans* (Jolivet, 2003), and *Halobacterium* sp. (Kotemann, 2005). The scattered appearance of ionizing-radiation resistance among distinct prokaryotic lineages indicates two possibilities. First, radioresistance could be a vestige of DNA-repair mechanisms that were present in ancestral species and have been retained in those organisms that continue to require this phenotype. This explanation assumes that the ancestor's ability to cope with DNA damage has been lost by most descendants, and predicts that the molecular mechanisms of radioresistance should be similar among ionizing-radiation-resistant species. Second, given the infrequent occurrence of ionizing-radiation resistance, it is possible that this phenotype has arisen in unrelated species through horizontal gene transfer, or possibly convergent evolution (Cox and Battista, 2005).

The genus name — *Deinococcus* — was based on the Greek adjective 'deinos', which means strange or unusual; an apt description for an organism with an ability to survive excessive DNA damage that sets it apart from much of the life on Earth. Members of this genus are unique and are characterised by their ability to survive high doses of ionising radiation as well as non-ionising radiation.

1.1. Distribution and phylogeny of *Deinococcus*

Deinococcus radiodurans R1 was the first radiation resistant bacterium isolated from spoiled canned meat (Anderson et.al., 1956). Till the beginning of this decade only seven members of this genus were reported while today the genus has more than 40 members and several 16S rDNA clone affiliations. Due to its pigmentation, gram positive nature initially *Deinococcus* were assigned to the genus *Micrococcus*. The family *Deinococcacae*, differentiated on the basis of morphology, consists of the *Deinococcus* and rod –shaped *Deinobacter*, represented by the only representative, *D. grandis*. On the basis of 16S rRNA gene sequence it was shown that deinococci formed a coherent group with the *Thermus* representing an ancient lineage in the Domain *Eubacteria* (Weisburg et al., 1989; Rainey, et al., 1997) (Fig. 1.1). Deinococci have been isolated from different niches from ordinary environment to stressed environments, a vast majority of which belong to desiccated environments. Table 1.1 lists the distribution of all the *Deinococcus* spp. that has been isolated till date with the source from which they were obtained (Slade and Radman, 2011).

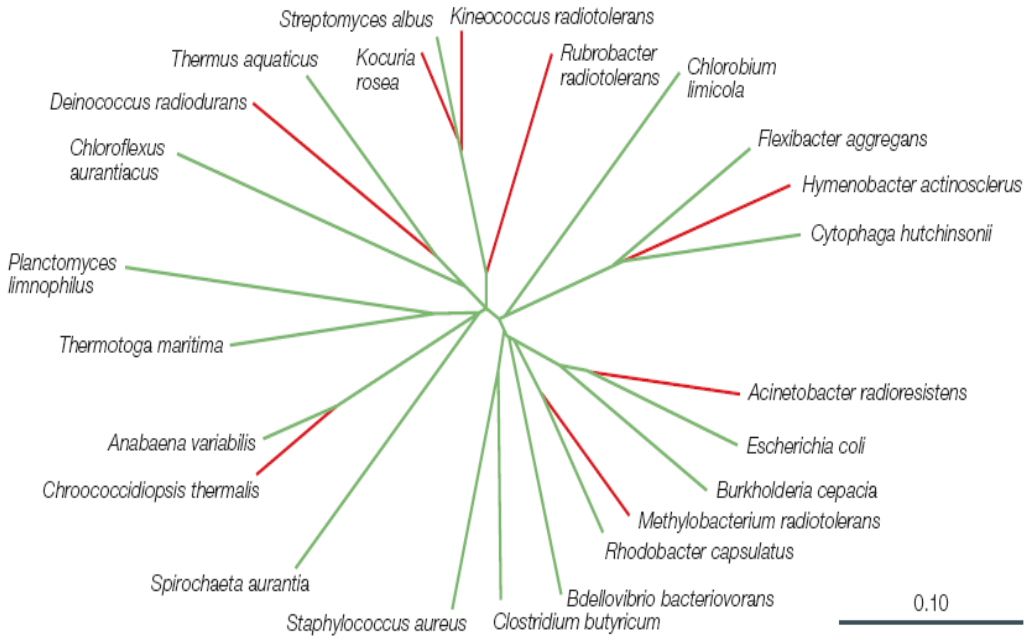


Fig. 1.1: Phylogenetic distribution of radiation–resistant Eubacteria (Cox and Battista, 2005). The red line depicts radiation resistant taxa**.**

Table 1.1: Source of type strains of Deinococcus species isolated till date.

S. No.	*Deinococcus* species	Source	Reference
1.	*D. radiodurans* DSM 20539 ^{T}	Gamma-irradiated canned meat	Anderson et al., 1956
2.	*D. radiopugnans* ATCC19172 ^{T}	Haddock tissue	Davis et al. 1963
3.	*D. radiophilus* DSM 20551T	Mumbai duck	Lewis et al., 1971
4.	*D. proteolyticus* DSM 20540 ^{T}	Faeces of a llama	Kobatake et al., 1973
5.	*D. grandis* DSM 3963^{T}	Faeces of an elephant	Oyaizu et al., 1987
6.	*D. geothermalis* AG-3a^{T}	Hot spring	Ferreira et al., 1997
7.	*D. murrayi* ALT-1b^{T}	Hot springs	Ferreira et al., 1997
8.	*D. indicus* Wt/1a ^{T}	Groundwater	Suresh et al., 2004
9.	*D. frigens* AA692 ^{T}	Antarctic soil	Hirsch et al., 2004
10.	*D. saxicola* AA1444 ^{T}	Antarctic sandstone	Hirsch et al., 2004
11.	*D. marmoris* AA63 ^{T}	Antarctic marble	Hirsch et al., 2004
12.	*D. hohokamensis* KR-40 ^{T}	Sonoran desert soil	Rainey et al., 2005
13.	*D. navajonensis* KR-114 ^{T}	Sonoran desert soil	Rainey et al., 2005
14.	*D. hopiensis* KR-140 ^{T}	Sonoran desert soil	Rainey et al., 2005
15.	*D. apachensis* KR-36 ^{T}	Sonoran desert soil	Rainey et al., 2005
16.	*D. maricopensis* LB-34 ^{T}	Sonoran desert soil	Rainey et al., 2005
17.	*D. pimensis* KR-235 ^{T}	Sonoran desert soil	Rainey et al., 2005
18.	*D. yavapaiensis* KR-236	Sonoran desert soil	Rainey et al., 2005
19.	*D. papagonensis* KR-241 ^{T}	Sonoran desert soil	Rainey et al., 2005
20.	*D. sonorensis* KR-87 ^{T}	Sonoran desert soil	Rainey et al., 2005
21.	*D. deserti* VCD115 ^{T}	Sahara desert sand	de Groot et.al., 2005
22.	*D. ficus* CC-FR-10 ^{T}	Rhizosphere of *Ficus religiosa*	Lai et al., 2006
23.	*D. mumbaiensis* Con-1 ^{T}	Contaminated agar plate	Shashidhar and Bandekar, 2006
24.	*D. peraridilitoris* KR-200 ^{T}	Coastal desert	Rainey et al., 2007
25.	*D. radiomollis* PO-04-20-132 ^{T}	Alpine environments	Callegan et al., 2008
26.	*D. claudionis* PO-04-19-125^{T}	Alpine environment	Callegan et al., 2008
27.	*D. altitudinis* ME-04-32 ^{T}	Alpine environment	Callegan et al., 2008
28.	*D. alpinitundrae* ME-04-04-52 ^{T}	Alpine environment	Callegan et al., 2008
29.	*D. aquaticus* PB 314^{T}	Freshwater	Im et al., 2008
30.	*D. caeni*Ho-08 ^{T}	Activated sludge	Im et al., 2008
31.	*D. aquatilis* CCUG 53370 ^{T}	Water	Kampfer et al., 2008
32.	*D. aquiradiocola* TDMA-uv53 ^{T}	Radioactive site	Asker et al., 2009
33.	*D. xinjiangensis* X-82 ^{T}	Desert soil	Peng et al., 2009
34.	*D. gobiensis* I-O ^{T}	Gobi desert	Yuan et al., 2009
35.	*D. aerius* TR-0125 ^{T}	High atmosphere	Yang et al., 2009
36.	*D. piscis* 3ax ^{T}	Marine fish	Shashidhar and Bandekar, 2009
37.	*D. aetherius* DSM 21230 ^{T}	Stratosphere	Yang et al., 2010

38.	*D. aerolatus* JCM 15422 ^{T}	Air	Yoo et al., 2009
39.	*D. aerophilus* JCM 15443 ^{T}	Air	Yoo et al., 2009
40.	*D. wulumuquiensis* NBRC 105665 ^{T}	Radiation-polluted soil	Wang et al., 2009
41.	*D. xibeiensis* NBRC 105666 ^{T}	Radiation-polluted soil	Wang et al., 2009
42.	*D. guangriensis* JCM 15082 ^{T}	Radiation Centre	Sun et al., 2009
43.	*D. depolymerans* TDMA-24 ^{T}	Radioactive freshwater site	Asker et al., 2010

1.2 General features of *Deinococcus radiodurans*

Deinococcus radiodurans R1 has been the major model of study for radiation resistance. The genome of *D. radiodurans* R1 (ATCC BAA-816) has been sequenced. The *D. radiodurans* chromosome is 3.28 Mb, with a GC content of 66.6%. The genome is segmented and consists of a 2.64 Mb chromosome (chromosome I), a 0.41 Mb chromosome (chromosome II), a 0.18 Mb megaplasmid and a 0.045-Mb plasmid (White et al., 1999). The members of the genus *Deinococcus* are exceptionally resistance to radiation, ionising (X rays and γ rays) as well as non-ionising radiation (ultravoilet, UV), oxidising agents as H_2O_2 (OH• generator) and paraquat (O_2• generator) as well as several mitogenic agents as mitomycin C (MMC).

There are two types of ionizing radiation, both produced by the decay of radioactive elements: electromagnetic (X and gamma radiation) and particulate (α and β particles) (Cox and Battista, 2005). Gamma rays are photons that generate ions, which react with other molecules to produce free radicals. Reaction with water molecules gives rise to hydroxyl radicals (OH•), the most reactive oxygen species (ROS) (Imaly, 2003; Ghosal et al., 2005). Fig. 1.2a depicts the ionisation effects of the different forms of ionising radiation. Ionizing radiation generates multiple types of DNA damage: base damage, SSBs, DSBs, and interstrand cross-links (Fig. 1.2b). DNA bases are most affected, with more than 80 different types of structural modifications induced by ionizing radiation. Approximately 10% to 20% of the time, the sugar-phosphate moiety is affected, which can lead to a single-strand break (Bjellard and Seeberg, 2003). On average, for every 20 SSBs induced by gamma rays in DNA, there is 1 DSB (Slade and Radman, 2011). If not repaired, DSBs prevent the replication of genomes and lead to cell death. Radiation-resistant and radiation-sensitive species have remarkably similar numbers of DSBs per Gy per genome

(0.002 to 0.006 DSBs/Gy/Mbp) (Gerard et al., 2001; Rothkamm and Lobrich, 2003) but differ in the amounts of oxidative DNA base damage (Kish et al., 2009). Fig. 1.2c shows that *D. radiodurans* can endure approximately 160 DSBs/ haploid genome without any mutation frequency whereas the radiation sensitive organism like *E. coli* shows 90% killing with about 6 DSB in its genome. Table 1.2 lists the D_{10} value for radiation resistant strains and DSBs caused by IR in the organisms listed.

D. radiodurans is extremely resistant to UV-C radiation (100 to 295 nm) and can efficiently repair UV-induced bipyrimidine photoproducts (BPPs), cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4 PPs) (Blasius et al., 2008). The major BPP in UV-irradiated *D. radiodurans* is CPD (47.2%), while 6-4 PPs are least represented (Moeller et al., 2010). Following 500 J/m^2 of radiation, more than 80 % of thymine-derived photoproducts are removed from *D. radiodurans* cells within 90 min (Blasius et al., 2008; Cox and Battista, 2005) and appear in the form of di- and trinucleotides in the medium outside the cells (Battista, 2005).

1.2.1 Physical structure of Deinococcus radiodurans

Although *D. radiodurans* is gram positive, the cell envelope is reminiscent of gram-negative bacteria due to its multilayered structure and lipid composition. The cell envelope of *D. radiodurans* is unusual in terms of its structure and composition. At least six layers have been identified by electron microscopy, with the innermost layer being the plasma membrane. A few strains of *Deinococcus* also exhibit a dense carbohydrate coat. Only the cytoplasmic membrane and the peptidoglycan layer are involved in septum formation during cell division. Fig. 1.3b shows the distribution of the layers of deinococcal cell wall (Rofthussus et al., 2006). The diamino acid L-ornithine found in the mucopeptide is the signature amino acid of the genus *Deinococcus* (Murray, 1986).

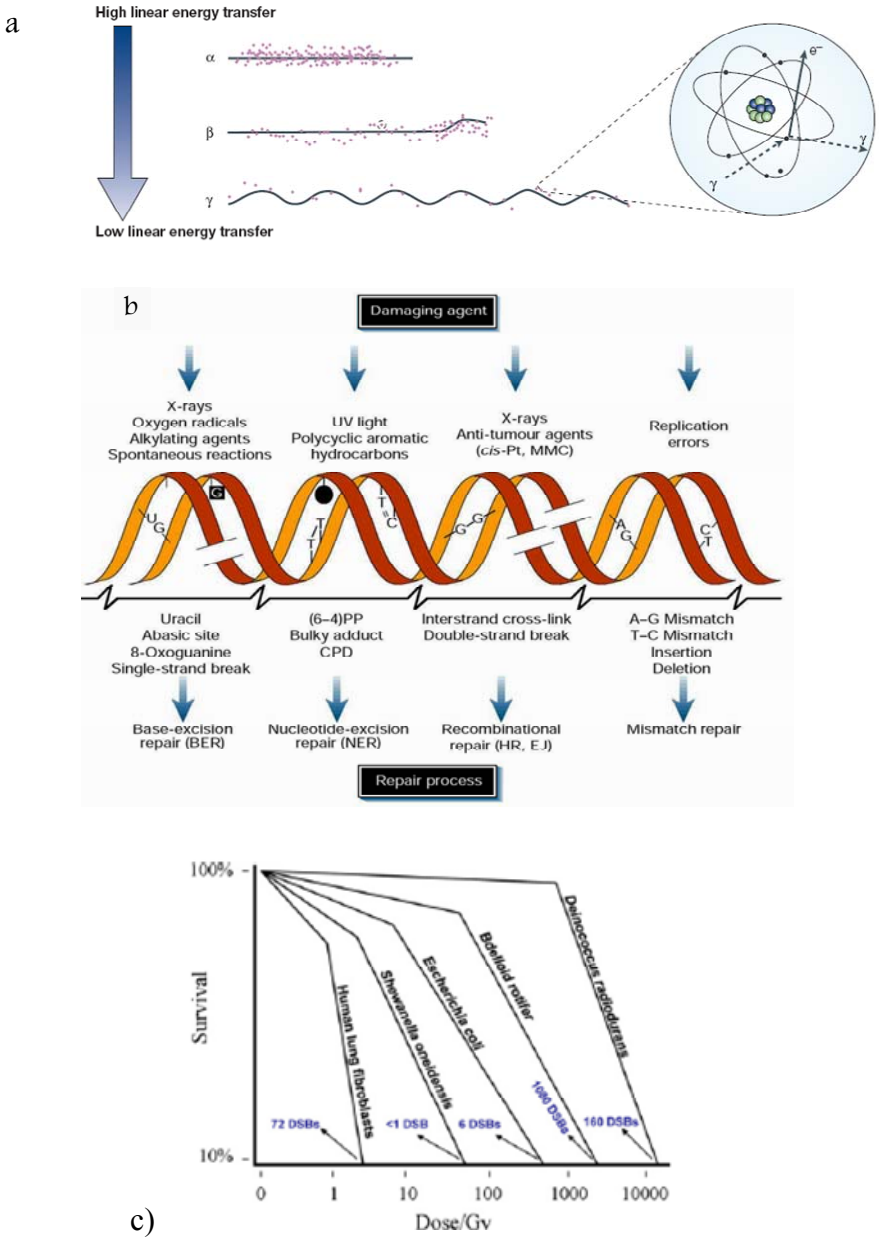


Fig. 1.2 Types of ionising radiation and their effect of DNA and survival of organisms. a) The tracks of three different types of ionizing radiation. Small dots indicate energy deposition events (Cox and Battista, 2005); b) Types of DNA damage by different DNA damaging agents, including radiation, and repair processes involved; c) Survival curves of representative organisms exposed to γ radiation. DSBs inflicted per haploid genome at D_{10} are indicated by arrows Daly, 2011).

Table 1.2 Comparative account of radiation resistance in bacteria

Strain	Genome size[a]	D_{10} (kGy)	DSB/Gy/Mbp (approximate linear density of DSBs in vivo)	Mn/Fe ratio	Reference
Deinococcus radiodurans	3.28	16	0.003	0.24	Ghosal et al., 2005
D. geothermalis	3.23	10	ND	0.46	Ghosal et al., 2005
Truepera radiovictrix	3.26	5.0[b]	ND	ND	Alberqueque et al., 2005
Kineococcus radiotolerans	4.76	2.0	ND	0.087	Bagwell et al., 2008
Enterococcus faecium		2.0	ND	0.17	Daly et al., 2004
Escherichia coli	4.64	0.7	0.006	0.0072	Ghosal et al., 2005
Pseudomonas putida	6.18	0.25	ND	<0.0001	Ghosal et al., 2005
Shewanella onedensisi	5.13	0.07	0.002	0.0005	Ghosal et al., 2005
Archeal isolates					
Halobacterium salinarum	5.2	5.0	0.002	0.19	Robinson et al., 2011
Thermococcus radiotolerans	2.05	8	ND	ND	Jolivet et al., 2004
Pyrococcus furiousus	1.91	2.5[c]	0.007	ND	Gerard et al., 2001

[a]*http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = genomeprj & cmd = Retrieve & dopt = Overview & list_uids = 65.*

[b]D_{60} dose that causes 40 % killing.

[c] D_{75} dose that causes 25 % killing.

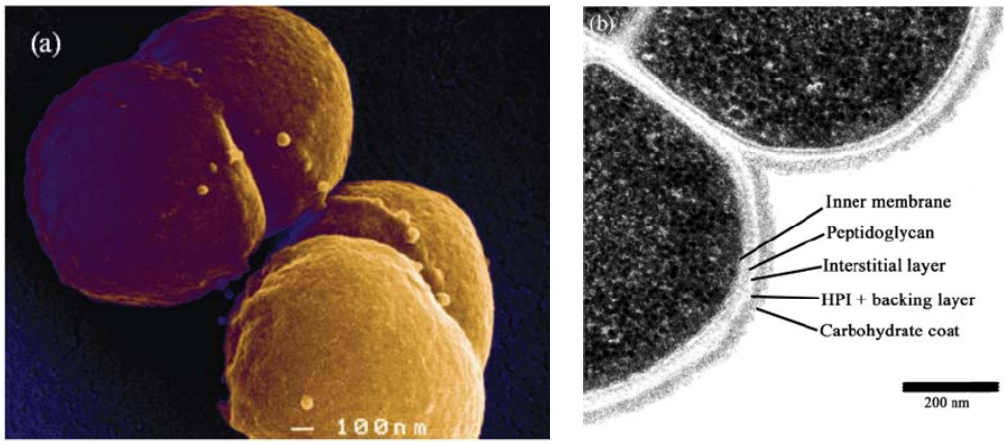


Fig. 1.3: Scanning electron micrograph of *Deinococcus radiodurans* R1. a) cells; b) cell wall (Rothfuss et al., 2006).

1.2.2 Metabolic configuration of Deinococcus radiodurans

D. radiodurans is an organotrophic bacterium with a proteolytic life-style (Ghosal et al., 2005). Amino acids are a preferred primary carbon energy source (He, 2009; Zhang et al., 2000), while carbohydrates are preferred in the following order: fructose > pyruvate > lactate >glucose> oxaloacetate > glycerol (Venkateswaran et al., 2000). *D. radiodurans* is dependent on exogenous nicotinic acid because it lacks key enzymes for NAD biosynthesis (Holland et al., 2006). Its methionine auxotrophy can be alleviated with vitamin B12, which is required as a cofactor for methionine synthase (Holland et al., 2006). In the presence of vitamin B12, sulphate can be used as the sole sulphur source (Holland et al., 2006).

Several metabolic properties help *D. radiodurans* to surmount oxidative stress: (i) proteolysis and the import of exogenous peptides and amino acids(Zhang et al,2000; Ghosal et al, 2005;), (ii) the conversion of glucose via the Pentose phosphate Pathway (PPP) into precursors for deoxyribonucleoside triphosphates (dNTPs) (Zhang et al.,2003), (iii) the suppression of ROS production by the induction of the glyoxylate bypass of the tricarboxylic acid (TCA) cycle and a reduction in the number of respiratory chain enzymes and enzymes with iron-sulphur clusters (Daly et al., 2010; Makarova et al., 2007), (iv) metabolic defects resulting in metabolite accumulation, and (v) carbohydrate and polyphosphate storage granules(Daly et al., 2010).

Interestingly, *D. radiodurans* R1 has been shown to have absolute requirement for Mn^{2+} to support normal growth in minimal medium (Daly et al., 2004). It has been shown to accumulate Mn^{2+} as reflected in higher Mn/Fe ratio as compared to the

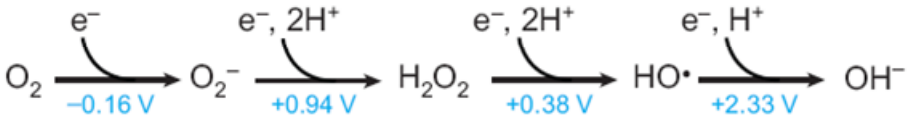
radiation sensitive bacterial strains (Table 1.2) (Daly et al., 2004; Ghosal et al., 2005). The addition of Mn^{2+} to the stationary phase culture of *D. radiodurans* R1 has been demonstrated to initiate fresh rounds of replication (Chou and Tan, 1990). Amendment of Mn^{2+} to the medium shifts the mode of glucose metabolism from PPP to TCA, making the cells sensitive to UV radiation (Zhang et al., 2000; Zhang et al., 2003).

1.3 Cellular damage caused by radiation

The central dogma of radiation biology is that the cytotoxic and mutagenic effects of radiation are the result of DNA damage principally by indirect effects mediated by HO• (Ghosal et al., 2005; Daly et al., 2007). Water is the most abundant chemical found in living cells and the primary ROS which arise during the radiolysis of H_2O are HO•, $O_2^{\bullet-}$

$$H_2O \xrightarrow{\text{Radiolysis}} OH\bullet + H^+ + e^-_{aq}$$
$$2\,OH\bullet \longrightarrow H_2O_2$$
$$O_2 + 2e^-_{aq} \longrightarrow O_2^{\bullet-}$$

Oxygen species are small molecules that cannot easily be excluded from active sites, and if they contact redox cofactors at a lower potential than themselves, then electron transfer can occur.



Reactions of this type are responsible both for the formation of ROS, that majorly consists of O^{2-} and the $HO^{\bullet}$, also for their subsequent inactivation of enzymes. The only oxygen species that can directly damage most biomolecules is $HO^{\bullet}$. Several transition metals such as Fe, Cu, Cr, V can directly catalyse the Fenton type chemistry to produce the reactive $HO^{\bullet}$, while other metals as Cd, Hg, Pb are known to produce ROS albeit indirectly by either replacing essential metals from their active centres as in case of Cd or depletion of the sulfhydryl group that reduces the reducing power of the cell. (Stohs and Bagchi, 1995). The hydroxyl radical oxidizes most organic molecules at diffusion-limited rates. While the Fenton reaction has been linked to protein carbonylation and membrane peroxidation, its most significant impact is likely to be upon DNA, since even a single DNA lesion is potentially mutagenic or lethal (Imlay, 2003; Imlay, 2008).

The fact that DSBs caused by radiation are essentially the same in bacterial genomes indicates that the target of radiation imposed damage is not limited to just DNA and other bio molecules are equally vulnerable targets of the radiation inflicted damage. Recently, fresh insight into the reparability of DSBs was gained by comparisons of DNA and protein damage in irradiated bacteria which have very different antioxidant levels and resistances. For a given dose of ionizing radiation, DSB lesion-yields were very similar, but protein oxidation lesion-yields were quantitatively related to survival (Daly et al., 2007; Daly, 2010; Krisko and Radman, 2010).

1.3.1 Protein oxidation

Carbonylation is the most common oxidative modification of proteins, often used as a biomarker of oxidative stress and has been demonstrated to be the cause of the radiation induced damage to the cell. Protein Carbonylation (PC) content in irradiated and unirradiated cells of DR1 is lower than those determined for radiation sensitive organisms (Daly et al., 2007). The accumulation of oxidative damage to proteins alters their catalytic activities and interactions, which leads to the disruption of cellular functions and culminates in cell death (Nystrom, 2005; Slade and Radman, 2011). The oxidation of DNA repair proteins causes error-prone activities, which result in DNA mutations (Daly et al., 2007).

Carbonyl derivatives are formed by a direct metal catalysed oxidative (MCO) attack on the amino-acid side chains of proline, arginine, lysine, and threonine. In addition, carbonyl derivatives on lysine, cysteine, and histidine can be formed by secondary reactions with reactive carbonyl compounds on carbohydrates (glycoxidation products), lipids, and advanced glycation/lipoxidation end products. The quantitatively most important products of the carbonylation reaction are glutamic semialdehyde from arginine (Fig. 1.4) and proline, and aminoadipic semialdehyde from lysine. Compared to other oxidative modifications, carbonyls are relatively difficult to induce and in contrast to cysteine disulphide bond formation, carbonylation is an irreversible oxidative process. Thus, a cell must rid itself of carbonylated proteins by degrading them (Nystrom, 2005). Carbonylation of proteins may occur during an increased ROS production, diminished ROS defence, or reduced protease activity (Frederickson et al., 2004; Avery, 2011).

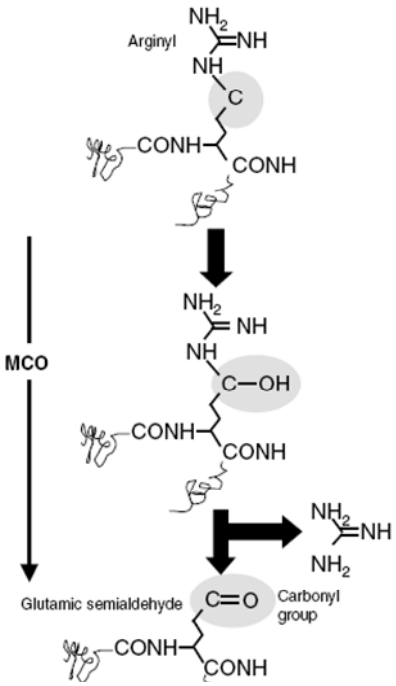


Fig. 1.4: Carbonylation process in proteins (Nystrom, 2005).

1.3.2 Membrane damage

Biological membrane serves as impermeable barriers and function in cellular transport processes, therefore severe membrane dysfunction is usually associated with loss of viability. Among the ROS, the protonated form of the superoxide anion and the hydroxy radical commonly initiate the process of autocatalytic lipid peroxidation (Imlay, 2003). Transition metals also catalyse lipid peroxidation. It is likely that a lipid peroxidation chain reaction begins after hydrogen abstraction from an unsaturated fatty acid to form a lipid radical. The lipid radical (L) thus formed reacts with molecular oxygen to form a lipid peroxyradical (ROO^-). The reaction is perpetuated when the lipid peroxy radical attacks another unsaturated fatty acid and abstracts a hydrogen atom to form a fatty acid hydroperoxide (ROOH) and perpetuate the initial reaction. The hydroperoxides thus formed will break down thermally or in the presence of O_2 or reduced transition metals to form lipid peroxy radicals (LOO^-) or lipid alkoxy radicals (LO^-), both of which can initiate new rounds of peroxidation (Farr and Kogoma, 1991).

Lipid alkoxy radicals can undergo cleavage of C-C bonds to form unsaturated fatty acid aldehydes and alkyl radicals. In addition to producing fatty acyl chains that are shorter than the parent chain, the end products of lipid peroxidation include alkanes, ketones, epoxides, and aldehydes. The net result of lipid peroxidation is

conversion of unsaturated lipids into polar lipid hydroperoxides, which can cause increased membrane fluidity, efflux of cytosolic solutes and loss of membrane protein activities. Extensive lipid peroxidation has been correlated with the ultimate disintegration of membrane integrity and cell death, but it has rarely been resolved whether it is a cause or effect of death. The rate of fatty acid peroxidation is directly proportional to the number of unsaturated C=C bonds. Transition metals have been reported to cause lipid peroxidation in bacteria (Avery, 2011).

1.3.3 DNA damage

Cellular exposure ionizing radiation results in numerous types of DNA lesions. In addition to the DNA damage caused directly by oxygen radicals, intermediate organic radicals that are formed during the propagation steps of lipid peroxidation can react with DNA causing strand breaks. Strand breaks and other lesions that block replication are likely to contribute more toward lethality than base damage that does not hinder replication, although the latter may contribute significantly to mutagenesis (Farr and Kogoma, 1991). Simultaneous inactivation of functions involved in BER (base excision repair) and in NER (nucleotide excision repair) yield strains that are sensitive to lethal mutagens, presumably via oxidative DNA lesions (Kuzmin et.al, 2005). At the same time, DNA repair-related mutants are ROS sensitive, linking this lethality to oxidative DNA damage including gross chromosomal rearrangements and instability (Avery, 2011; Imlay and Linn, 1988; Hasset and Cohen, 1989). In *E. coli*, DNA may be a more important ROS target where membrane lipid oxidation is less likely (Avery, 2011). In cases where lethal DNA damage is linked to pro-oxidant toxicity, the primary target can in fact be protein(s) required for preserving DNA integrity. Here, elevated DNA damage is a secondary outcome of direct protein inactivation (Imlay and Linn, 1988). Finally, DNA damage itself can result in elevated ROS generation, with the potential to attack other targets which may be more pivotal for cell viability (Avery, 2011).

1.4 Radiation resistance mechanism in *Deinococcus radiodurans* R1

The remarkable capacity of *D. radiodurans* R1 to withstand ionising and non-ionising form radiation has attracted the major deinococcal research. It can also withstand several DNA mutagenic agents the repair mechanism of which overlaps the mechanism involved in radiation resistance.

1.4.1 Resistance to UV-C Radiation

D. radiodurans possesses the classical nucleotide excision repair pathway (UvrABC) for the removal of pyrimidine dimers. It involves a protein complex (UvrABC exonuclease) that recognizes the structural changes in DNA caused by UV damage and creates the dual incisions 5´and 3´ to the damaged site. The UV damage endonuclease (UVDE) pathway (Minton, 1994; Mosley and Evans, 1983) is mediated by endonuclease (*uvsE*), which has a novel requirement for manganese ions and an endonucleolytic mode of action that is different from that of UvrABC (Evans and Mosley, 1983; Mosley and Evans, 1983).

The two pathways have overlapping functions, as both needs to be inactivated to produce a UV sensitive phenotype (Mosley and Evans, 1983). An *uvrA uvsE* double mutant is 100 fold more sensitive to 250 J/m^2 UV than the wild type and loses the shoulder of UV resistance (Earl et al., 2002). The slightly higher UV sensitivity of the *uvrA* mutant than the *uvsE* mutant suggests that UvrABC is more important for UV resistance than is UVDE (Slade and Radman, 2011). In addition, UvrABC is constitutively expressed which indicates that UvrABC is important for the continuous removal of damaged nucleotides from the cells (Lipton et al., 2002). Both the UvrABC and UVDE pathways require Pol I, as the *polA* mutant is equally sensitive to UV radiation as the *uvrA uvsE* double mutant (Gutman et al., 1993).

The recombination-deficient *recA* mutant is more sensitive to UV radiation than is the *uvrA uvsE* mutant, which suggests that recombinational repair is more significant than the two excision repair pathways for UV radiation resistance in *D. radiodurans* (Tanaka et al., 2005). UV sensitive phenotypes are also produced due to mutations in other recombinational genes, *recO* and *recF*, (Chang et al., 2010; Xu et al., 2008). Unlike ionizing radiation, UV does not induce point mutations in *D. radiodurans*, even at doses as high as 1,485 J/m^2 (Tanaka et al., 2005). The absence of translesion synthesis (TLS) DNA polymerases in *D. radiodurans* (Makarova et al., 2001) contributes to the high fidelity of the repair of UV lesions.

1.4.2 Resistance to Ionising Radiation (IR)

D. radiodurans R1 can sustain gamma irradiation doses that introduce hundreds of double-strand breaks in its genome. The kinetics of DNA double-strand break repair is very rapid as an intact genome complement is reconstructed from a myriad of fragments in few hours (Fig. 1.5a) (Blasius et al., 2008).

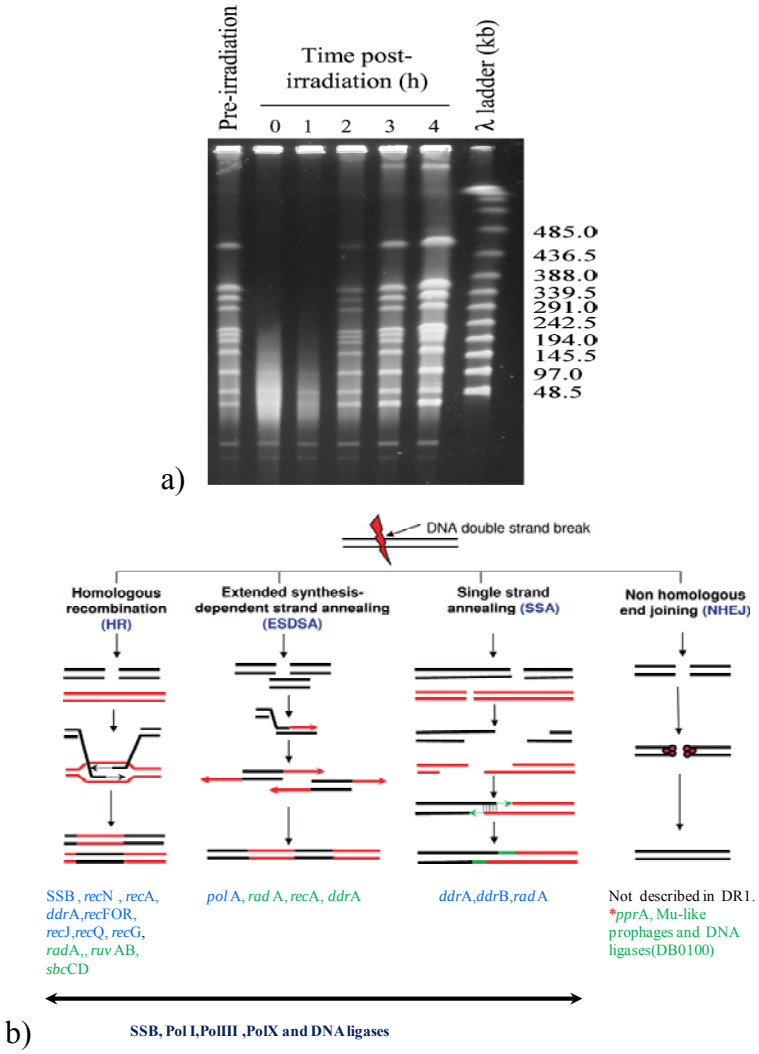


Fig. 1.5 Kinetics and repair of DSB in *D. radiodurans*. a) Kinetics of DNA DSBs in cells post-γ –irradiation and analysed by PFGE; b) Different pathways for DSB in *D. radiodurans* R1. Genes involved in each of the pathways are indicated in blue while of the proposed function but not experimentally demonstrated are shown in green. Common proteins involved in DSB repair is indicated below the arrow (Blasius et al., 2008).

Several mechanisms have been proposed to account for such an efficient repair (Figure 1.5b). Homologous recombination (HR) in *D. radiodurans* R1 forms a bulk of repair post-irradiation and involves essentially the same proteins that are employed in *E. coli* recombinational repair. In *D. radiodurans* R1, HR can be divided into RecA dependent and RecA independent pathway recovery. Major proteins involved in the recombinational repair in *D. radiodurans* R1 are listed in Table 1.3.

Table 1.3: Role of major proteins involved in recombinational repair in D. radiodurans R1 (compiled from Slade and Radman, 2011)

Gene	Function	Phenotype of the mutant	Reference
*rec*J (DR1226)	5′–3′ single- strand-specific exonuclease activity, producing 3′ends	Modestly sensitive to gamma rays and only slightly sensitive to UV and H_2O_2	Bentchikou et al., 2010 Cao et al., 2010
*uvr*D (DR1775)	Major helicase	Moderately sensitive toγ radiation. Delayed DNA synthesis and reassembly.	Bentchikou et al., 2010
recQ (DR1289)	Helicase unwinds DNA 3′–5′	No effect on radiation resistance but highly sensitive to MMC, UV and H_2O_2	Bentchikou et al., 2010 Huang et al., 2007
*rec*N (DR1477)	Tethers DNA molecule in a cohesion-like fashion and prevents the separation of DNA	Sightly increased sensitivity to gamma rays, UV radiation, and MMC	Funayama, T., et al., 1999
rec FOR *rec*F (DR1089) *rec*O (DR0819) *rec*R (DR0198)	loads RecA onto the 3′-tailed DNA coated with SSB	Extremely sensitive to gamma rays, and display incomplete genome reconstitution, a reduced level of DNA breakdown, and absence of DNA synthesis.	Xu et al., 2008. Bentchikou et al., 2010
*rec*A (DR 2340)	Homologous recombination	Reduced γ, UV and MMC resistance	Slade and Radman, 2011
*rad*A (DR1105)	assist RecA in priming DNA repair synthesis during ESDSA	moderately sensitive to ionizing radiation and have a delay in repairing.	Slade et al., 2009, Zhou et al., 2006
*ruv*A (DR1274),	RuvAB complex stimulates the branch migration of Holliday junctions in the 5′-to-3′ direction	ND	Tsaneva et al., 1993
*ruv*B (DR0596)		modestly sensitive to UV radiation, gamma rays, and MMC	Kityama et al., 1997
rec G (DR1916)	branch migration of Holliday junctions in 3′-to-5′ direction	highly sensitive to gamma rays and H_2O_2	Whitby et al., 1993.
*rec*D (DR1902)	helicase activity with 5′ -3′ polarity with low processivity	enhances the efficiency of transformation by exogenous homologous DNA and has anti-recombinogenic properties.	Shadrick and Julin , 2010
Novel *Deinococcus* repair protein			
*ppr*A (DRA0346)	stimulates DNA end-joining reactions catalyzed by ATP- and NAD-dependent DNA ligases	highly sensitive to ionizing radiation, MMC and UV-A radiation	Narumi et al., 2004; Bauermeister, 2009
DdrA (DR0423)	protects 3′ ssDNA overhangs from degradation by *E. coli* exonuclease		Harris et al., 2004; Omelchenko et al., 2005

1.4.2. a. recA dependent pathway

The recombinational repair of double strand breaks (DSBs) in *D. radiodurans* proceeds via two homologous recombination processes, extended synthesis dependent strand annealing (ESDSA) and homologous recombination by crossovers, both of which rely on the RecA recombinase (Blasius et al., 2008; Cox and Battista, 2005). RecA and its homolog, RadA, prime DNA repair synthesis on partially overlapping fragments as templates (Slade et al., 2009; Zahradka, 2006)). RecA is essential, as RadA cannot replace RecA-mediated DNA synthesis priming. Following RecA-RadA catalysed priming, DNA Pol III initiates DNA repair synthesis (Slade et al., 2009). DNA repair synthesis generates long newly synthesized single strands, which processively dissociate from the migrating D loops, aided by DNA helicases, and can readily anneal with complementary strands. The 3′ flaps generated after the annealing of single strands could be incised by SbcCD. The long linear products of ESDSA require RecA-mediated crossovers within overlapping homologies to mature into circular chromosomes (Zahradka, 2006).

1.4.2.b. rec A independent pathway

In the absence of RecA, approximately one-third of the DSBs generated by ionizing radiation can be rejoined by a RecA-independent pathway (Slade and Radman, 2009; Zahradka et al., 2006). The RecA-independent single strand annealing (SSA) pathway may involve proteins such as DdrA, which protects 3′ ssDNA ends from degradation (Harris et al., 2004); DdrB, an SSB-like protein with strand-annealing properties (Norais et al., 2009; Sheng et al., 2005); and RadA, a distant RecA homolog (Slade et al., 2009). The lesser extent of DNA degradation observed for the *recA* mutant (Slade et al., 2009) is congruent with the importance of protecting the DNA fragments' ends in the absence of RecA before annealing with overlapping fragments can occur. In the absence of RecA, RadA also seems to contribute to the RecA independent pathway of DSB repair, although its role remains unclear (Slade et al., 2009).

1.5 Models of radiation resistance in *D. radiodurans* R1

Although DNA repair proteins in *D. radiodurans* R1are enzymatically very similar to those in other bacteria, their remarkable efficiency in assembling DNA fragments may be partially imparted by other features of the organism. Of the several hypotheses that have been forwarded for the radiation resistance in *D. radiodurans* R1 key hypothesis are as follows.

1.5.1 Chromosome alignment and nucleoid morphology facilitate genome reassembly

Several models explain how structural aspects may contribute to the observed rapidity and efficiency of the RecA mediated homology search in *D. radiodurans* R1: (i) genome condensation, (ii) ring-like nucleoid morphology, (iii) DNA-membrane association, and (iv)chromosome alignment. Absolute role of any one single physical attribute has not been established. This model made two major predictions: first, *recA*-dependent recombination between homologous DSB fragments originating from widely separated genomic locations should show strong positional effects on irradiation and, second, transmission electron microscopy (TEM) of chromosomal DNA in *D. radiodurans* should reveal evidence of structures linking chromosomes. Both predictions were tested and refuted: molecular studies showed high levels of recombination between homologous DSB fragments irrespective of their genomic origin (Daly et al., 1994; Daly and Minton, 1995, 1996); and no linking structures were observed by TEM-based optical mapping (Lin et al., 1999). Another model proposed that high levels of chromosomal condensation observed in *D. radiodurans* grown in rich medium facilitated repair by holding proximal DSB ends together and that manganese promoted the condensation of its nucleoids into ring like structures (Levin-Zadman et al., 2003). This model is also generally discounted: *D. radiodurans* grown in defined minimal medium (DMM) did not display condensed nucleoids but remained extremely IR resistant and *D. radiodurans* that was depleted in manganese displayed condensed ring like nucleoids but was rendered IR sensitive (Daly et al., 2004; Ghosal et al., 2005). Thus, IR-induced DSB fragments in irradiated *D. radiodurans* are not immobilized and the structural form of its nucleoids does not play an important role in radioresistance

1.5.2 Subset of uncharacterized genes encode novel proteins that enhance the efficiency of DNA repair

Experimental evidence supporting that *D. radiodurans* relies, at least in part, on a core set of ordinary DNA repair proteins is now well established (Blasius et al., 2008; Cox and Battista, 2005; Makarova et al., 2007; Slade et al., 2009). Whole transcriptome studies on irradiated *D. radiodurans* were used to identify novel genes induced during recovery (Liu et al., 2003; Tanaka et al., 2004); there are only approximately 150 uncharacterized genes that are shared between the three *Deinococcus* genomes. Among those which were induced in irradiated *D.*

radiodurans, only few have a discernible functional relevance to the preservation of genome integrity. Another moderately IR-sensitive *D. radiodurans* mutant is *pprA*2, which is a putative DNA-binding protein (Kota and Misra, 2006). However, for most of the mutants derived from this subset of novel genes, there was no drastic change in the level of IR resistance, indicating that few of the putative resistance proteins, at least individually, make a substantial contribution to the recovery of irradiated *D. radiodurans*. Thus, functional genomics evidence supporting this hypothesis has grown progressively weaker (Makarova et al., 2007).

1.5.3 Manganese as protective agent against IR

Hydroxyl radicals are the primary reactive oxygen species (ROS) generated by IR and indiscriminately damage all macromolecules (Imlay, 2003). It has been proposed that naturally sensitive bacteria are killed by IR mainly owing to protein oxidation, whereas manganese complexes in extremely resistant bacteria protect enzymes needed to repair DNA and allow survival (Daly, 2010). This observation correlated well with the intracellular Mn/Fe ratio in the radiation resistant cultures. The role of accumulated manganese in the chemical removal of ROS has been ascribed to the formation of small complexes. Inorganic phosphate and Mn^{2+} form complexes that catalytically remove superoxide (Barnese et al., 2008) and amino acids and peptides form complexes with Mn^{2+} that catalytically decompose hydrogen peroxide (Berlett et al., 1990). The formation of Mn^{2+} complexes is highly dependent on the availability of inorganic phosphate and free amino acids or peptides and other small molecules. Thus, the strong trend in the *Deinococcus* genomes of genes encoding phosphatases, nucleases, and proteases are predicted to support the formation of Mn^{2+} complexes (Ghosal et al., 2005; Makarova et al., 2001, 2007). This hypothesis is strongly favoured with respect to the growing genetic and functional genomics.

1.6 Anti-oxidant protection in *D. radiodurans* R1

The oxidative damage to the cell is limited by ROS scavenging activity of the cell. The *D. radiodurans* antioxidant defence machinery is active against all three primary reactive oxygen species: hydroxyl radicals (OH•), superoxide radicals (O_2•), and hydrogen peroxide (H_2O_2). The following section briefly describes the repertoire of enzymatic and non-enzymatic anti-oxidant activity of *D. radiodurans* R1.

1.6.1 Enzymatic protection

D. radiodurans encodes three catalases, four superoxide dismutases (SOD) (Mn-dependent and Cu/Zn-dependent), a cytochrome *c* peroxidase and an iron-dependent peroxidase (Makarova, 2001). *D. radiodurans* is much more resistant to H_2O_2 than is *E. coli*, with a large shoulder in the survival curve (Wang and Schellhorn, 1995). According to data reported by Wang and Schellhorn (1995), the catalase activities during exponential and stationary phases are 127 and 32 times higher those in *E. coli*, respectively. Catalase activity is affected by H_2O_2 (Wang and Schellorn, 1995), ionizing radiation (Tanaka et al., 1996), the addition of manganese (Chou and Tan, 1990), and the growth phase (Wang and Schellorn, 1995), with a higher level of catalase activity in stationary phase cells than in exponential phase cells (Wang and Schellorn, 1995). Catalase activity is negatively controlled by the transcriptional regulator DrRRA (Wang et al., 2008) and positively controlled by OxyR (94). DR1998 is induced in response to ionizing radiation (Tanaka et al., 1996; Tanaka et al., 2004).

Among the SOD proteins, Mn-SOD is constitutively expressed (Lipton et al., 2002). It efficiently eliminates higher $O_2^{\bullet-}$ concentrations than Mn-SODs in *E. coli* and humans due to the more rapid protonation and release of H_2O_2 (Abreu et al., 2008). *D. radiodurans* catalase and superoxide dismutase mutants are sensitive to H_2O_2 and paraquat, respectively, but not to ionizing radiation at doses lower than 16 kGy (Markillie et al., 1999). The absence of a strong positive correlation between catalase activity and (i) the MIC of H_2O_2 or (ii) ionizing radiation resistance across *Deinococcus* species suggests that other (non-enzymatic) antioxidants (such as manganese complexes) contribute to the scavenging of H_2O_2 (Shashidhar et al., 2010). Fig.1.6 summarises the regulation of catalase and SOD and catalase in DR1

D. radiodurans also encodes other oxidative defence proteins, such as glutaredoxin, thioredoxin, thioredoxin reductase, and alkyl hydroperoxide reductase, while glutathione, glutathione reductase, and glutathione peroxidase are absent (White et al., 1999). In *E. coli*, the alkyl hydroperoxide reductase is the primary scavenger of endogenous H_2O_2 (Seaver and Imaly, 2001). Thioredoxin reduces oxidized cysteines in proteins and is reverted from its oxidized form by thioredoxin reductase in an NADPH-dependent reaction (Obeiro et al., 2010; Seo and Lee, 2006). *D. radiodurans* also possesses two peptide methionine sulfoxide reductases, MsrA and MsrB (Omelchenko et. al, 2005), which are important for the reduction of oxidized

methionine in proteins. MsrA is transcriptionally induced following ionizing radiation (Tanaka et al., 2004).

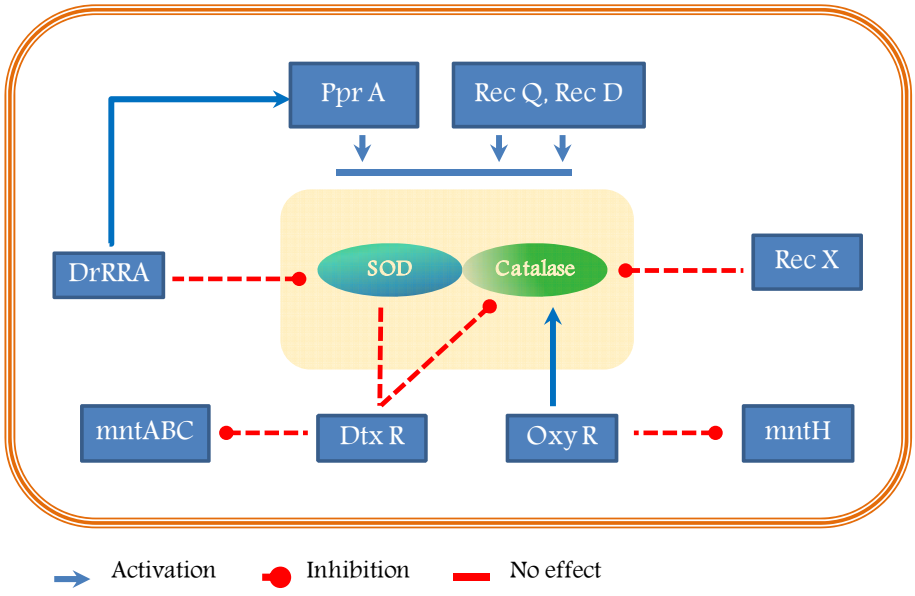


Fig. 1.6: Regulation of ROS combating enzyme in *D. radiodurans* R1. Compiled from Slade and Radman (2011)

1.6.2 Pyrroloquinoline–quinone (PQQ)

The role of *pqq*E was postulated as an inducer of a DNA repair and homologous recombination protein kinase, involved in radiation resistance and double strand break repair in DR1 (Rajpurohit et al., 2008). Heterologous expression of deinococcal pyrroloquinoline–quinone (PQQ), a redox factor for several dehydrogenases, in *E. coli* enhanced the catalase and SOD activity in the host *E. coli* (Khairnar et al., 2003). PQQ neutralizes the ROS by directly reacting with them through single electron transfer mechanism and the adducts, thus formed, are non-oxidant in nature. PQQ also functions in a concentration dependent manner in protecting the proteins and DNA from the oxidative damage caused by γ radiation in solution, suggesting a role of PQQ as a radioprotector. *pqq*E mutants of the DR1 and sensitive to IR as well as mitomycin C induced damage to DNA and exhibit a retarded recovery from radiation as opposed to the wild type cells (Misra et al., 2004).

1.6.3 Dps (DNA protection during starvation) proteins

The effect of Dps on survival of the cells recovering from high dosages of H_2O_2 suggests that DNA damage might be one of the sites of toxic lesions caused by high

concentrations of H_2O_2 as it is believed to be caused by Fe^{2+} tightly bound to the DNA bases and phosphodiester backbone (Martinez and Kolter, 1997). Thus, prevention of coordination of Fe^{2+} atoms with the DNA by Dps binding could explain protection from both direct and indirect (through Fenton's chemistry) mode of killing. Alternatively Dps could act by scavenging hydroxyl radicals in the vicinity of DNA. Such a mechanism has been proposed to explain the decreased sensitivity to oxidative DNA damage of chromatin. *D. radiodurans* encodes two Dps homologs, Dps1 (DR2263) and Dps2 (DRB0092). A dimeric form of Dps1 protects DNA from hydroxyl radical cleavage (Groove and Wilkinson, 2008), which may also be true for Dps2, as the *dps2* mutant is sensitive to H_2O_2 (Slade and Radman, 2011). Both Dps1 and Dps2 are induced in response to ionizing radiation (Liu et al., 2003; Tanaka et al., 2004).

1.6.4 Carotenoids

Most of the deinococci are pigmented, pigmentation ranging from red to pink to orangish-red. Deinoxanthine is the prominent carotenoid present in *D. radiodurans*. Deinoxanthin acts as more efficient scavenger of H_2O_2 and singlet oxygen than lycopene, β-carotene and lutein because of their extended conjugated double bonds (Tian et al., 2007). DcrtB, mutant of *D. radiodurans* wherein carotenoid biosynthesis was blocked, showed enhanced protein oxidation following treatment with H_2O_2 indicating that the intracellular proteins in the cell without carotenoids were more susceptible to oxidative damage compared to the wild-type cell (Tian et al., 2009).

1.6.5 Manganese complex and its effect on radiation induced oxidative stress

The ability of organic complexes of Mn^{2+} was demonstrated first by Berlett et al., (1990). The first report of Mn^{2+} accumulation in *D. radiodurans* was by Leibowitz et al. (1976), who demonstrated that *D. radiodurans* contained approximately 100 times more Mn than *E. coli* when grown in a defined minimal medium (DMM). Later Daly et al., (2004) established that all radiation resistant bacteria accumulated higher concentration of Mn^{2+} as opposed to Fe^{2+} and therefore reflected in higher Mn/Fe ratio. The same observation was also extended for desiccation resistant bacteria (Daly et al., 2004).

Compared to most organisms, proteins in *D. radiodurans* are highly protected from ROS, but lose their resistance when purified from the cells (Daly et al., 2007). In contrast, DNA in *D. radiodurans* R1 is damaged with essentially the same dose

dependence as in all prokaryotic and eukaryotic cells examined (Daly et al., 2004; Daly, 2009; Gladyshev and Meselson, 2008). When orthophosphate (13 mM), Mn^{2+} (200 mM), and peptides (3 mM) were combined *in-vitro* at concentrations approximating those in *D. radiodurans*, the mixture preserved the activity of *Bam* HI and glutamine synthetase exposed to 17.5 kGy, but did not significantly protect DNA. 17.5 kGy represents the outer limits of *D. radiodurans* survival and breaks its 4–8 haploid genomes per cell into 1,000–2,000 DSB fragments (Daly et al., 2011). Thus, protein protection mediated by small Mn^{2+} complexes provides an explanation for the large shoulders in ionizing radiation dose-response curves of *D. radiodurans* survival which distinguishes them from radiosensitive organisms (Daly et al., 2004).

Based on whole-genome comparisons, there is a remarkable abundance in DR1 of genes encoding catabolic enzymes including phosphatases, nucleases and proteases, which would be expected to give rise to the sorts of small molecules accumulated in the DR1 ultrafiltrate (Krisko and Radman, 2010; Daly et al., 2010).*D. radiodurans* exposed to ionizing radiation produces an intracellular pool of nucleotides which are subsequently converted to nucleosides (Battista, 1997) that form complex with Mn^{2+} that prevent PC during irradiation induced ROS.

Neutron activation analysis (NAA) reveals that, *D. radiodurans* R1 accumulated a total of approximately. $0.29610E^{-18}$ mol Mn/cell (approx. 1.861 Mn atoms/cell; or, 4 mM Mn, given a cell volume of $6.56mm^3$). When *D. radiodurans* R1 was incubated in minimal medium containing the radioisotope ^{54}Mn, the cells accumulated approximately 3mM Mn (Daly et al., 2004). X-ray fluorescence (XRF) microspectroscopy revealed that Mn^{2+} is distributed throughout DR1 cells grown in TGY, but with regional intracellular Mn^{2+} concentrations ranging from 0.4 to 3 mM (Daly et al., 2007). *D. radiodurans* lacks most of the Fe-chelating and Fe-transport systems identified in IR-sensitive bacteria (Ghosal et al., 2005; Makarova et al., 2007); most iron in *D. radiodurans* is sequestered outside of the cytosol in the septum between dividing cells (Fig. 1.7) (Daly et al., 2007).

Intracellular accumulation of Mn^{2+} and its complexation with small molecules such as the peptides, orthophosphate has been reported in *D. radiodurans* (Daly et al., 2009) and is forwarded as the main mechanism of radiation resistance in *Deinococcus*. Not only in *Deinococcus* but also in other bacteria Mn^{2+} play an important role in survival such as those in *Bacillus* spore formation, pathogenesis of

streptococci, and survival of facultative aerobes such as *Lactobacillus plantarum* (Archibald and Fridrovich, 1981).

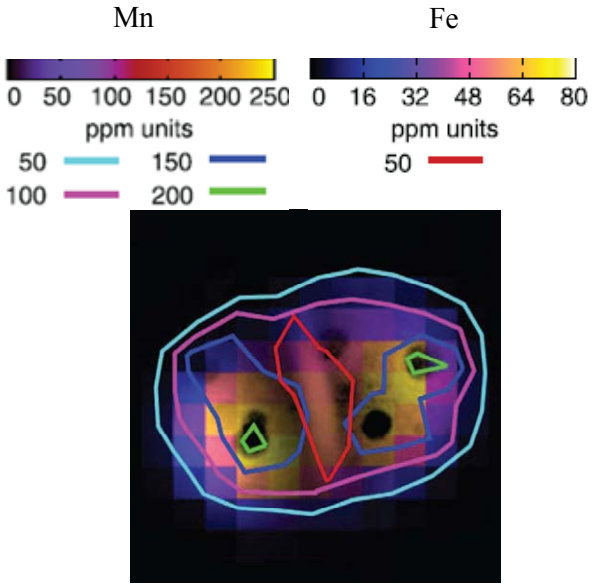


Fig. 1.7: XRF image analysis demonstrating the distribution of Mn^{2+} and Fe^{2+} in *D. radiodurans* R1 (Daly et al., 2007)

Manganese is the only metal involved in the water oxidizing complex of photosynthetic organisms (Kehres and Maguire, 2003), and involved in the enzymatic dismutation of superoxide radical anion. The discovery of Mn^{2+} as a substitute of SOD to scavenge O_2^- in *Lactobacillus* and *Neiserria* has diverted the attention to the importance of Mn^{2+} in bacterial systems (Jakubovics and Jenkinson, 2000). Some well-known protein that are Mn^{2+} dependent are summarised in Table 1.4.

1.7 *Deinococcus spp.* as candidate for bioremediation

Nuclear waste sites were generated during the cold war and continue to grow in number due to the use of nuclear power to generate electricity. In the United States alone , buried radioactive wastes is estimated to be cover an area of (3×10^6 m^3) that has contaminated about 7×10^7 m^3 of surface and subsurface soils and about 3×10^{12} dm^3 of groundwater. The most common contaminants from DOE wastes that have been found in ground and ground waters include the radionuclides ^{235}uranium (γ, α) E , ^{238}plutonium (α) E, ^{99}technetium (β^-) E, ^{90}strontium (β^-) E, and ^{137}cesium (γ, β^-)E, and the metals chromium, lead and mercury along with a myriad of toxic organic compounds (e.g. toluene and trichloroethylene (TCE) (Daly, 2000).

Table 1.4: Mn 2+ dependent pathways in bacteria (Jakubovics and Jenkinson, 2001)

Process /pathway	Enzyme /protein	Reference
	Mn^{2+} dependent deinococcal proteins	
DNA repair	UVDE endonuclease	Evans and Mosley, 1985
	DNA polymerase X	Blasius et al., 2006
	NAD dependent DNA ligase	Blasius et al., 2006
RNA repair	RNA ligase	Martins and Shumann, 2004
Nucleic acid metabolism	Nudix hydrolases	Fisher et al.,2006
Oxidative stress response	Mn-SOD	Juan et al., 1991
Sugar metabolism	Fructose-1,6 bisphosphate aldolase	Zhang et al., 2006
	Mn^{2+}dependent proteins in bacteria	
Photosynthesis	Mn stabilising protein(PSII-O)	Morgan et al., (1998)
Gluconeogenesis	PEP synthase Pyruvate Carboxylase	Chao et al., (1993) Mukhopadhay et al., (1998)
Glycolysis	3-Phosphoglyerate mutase	Chandler et al., (1998)
Sugar metabolism	6-Phospho-β-glucosidase L-Fucose isomerase	Thompson et al., (1999) Seamann & Schulz, (1997)
Amino acid metabolism	Arginase Glutamine synthetase Threonine 3- dehydrogenase	Sekowaka et al., (2000) Abell et al., (1995) Chen et al., (1995)
Peptide cleavage	Aminopeptidase P	Yocum & Pecoraro, 1999
Nucleic acid degradation	Ribo nuclease H III Endonuclease IV	Ohtani et al., 2000 Hosfeld et al., 1999
Signal transduction	Serine /threonine protein and phosphatases 1 and 2	Missiakas & Raina, 1997
Stringent response	(p)ppGpp3 Pyrophosphohydrolase	Rao et al., 1998
Oxidative stress response	Magani- catalase Mn-SOD	Whittaker et al., 1999 Fridovich, 1995

These vast waste sites are therefore potential targets for less expensive *in situ* bioremediation technologies utilizing specialized microorganisms that can detoxify both metallic and organic contaminants. However, the utility of microbiological methods for the primary treatment of highly radioactive environmental wastes will largely be determined by the ability of microorganisms catalyzing the desired

function(s) to survive and function under radiation stress and non-pathogenicity of the culture.

Several bacteria such as *Shewanella* spp., *Pseudomonas* spp. are well known for the capacity to reduce variety of metals and mineralize several organic compounds respectively but are radiation sensitive. Therefore for the cleanup of the nuclear waste sites the radiation resistant microorganisms become the obvious choice. Most radiation-resistant bacteria that have been reported are spore-formers and are not remarkably radiation resistant when growing vegetatively; many of them are pathogens such as *Enterococcus faecium* and *Alcaligenes* spp., and most of them lack a developed system for genetic manipulation (Daly, 2000). Bacteria belonging to the family *Deinococcaceae* are not only the most radiation-resistant organisms discovered, but they are vegetative, easily cultured, and non-pathogenic. Other radiation resistant bacteria that have been reported are not studied with respect to their potential as bioremediation. Although several radiation resistant bacterial isolates have been described, the ease of genetic manipulation of the members of genus *Deinococcus* further affirms the use of the deinococci for bioremediation of the radioactive waste sites.

1.8 Engineering *Deinococcus radiodurans* for bioremediation

Most of the deinococci can grow in presence of 6000 rad/h comparable to those found at several nuclear waste sites. The ability to grow in presence of chronic radiation and ease of transformability of *D. radiodurans* allows engineering for bioremediation at nuclear waste sites. The engineered strain of *D. radiodurans* R1 expressing MerA not only tolerates 30-50 μM Hg^{2+} but also reduces Hg^{2+} to elemental Hg° (Brim et al., 2000). Similarly, *D. geothermalis*, a thermophile, has been engineered with *mer*A and finds applicability at nuclear waste where the higher temperatures prevail (Brim et al., 2003). Quin et al., (2005) transformed *D. radiodurans* R1 with the metal binding domain of MerR, regulatory protein, to effectively increase the tolerance of the transformed strain to Hg^{2+}. Appukuttan et al., (2006) successfully transformed *D. radiodurans* R1 with *pho* N from local isolate of *S. enteritica* serovar *typhimurium* for precipitation of Uranium to Uranium phosphate from dilute nuclear waste. Apart from transforming *D. radiodurans* for metal remediation, it has been engineered with the toluene dioxygenase genes (*todC1C2BA*) of *P. putida*. During chronic irradiation, these strains were able to oxidize toluene, chlorobenzene, and 3, 4-dichloro-1-butene (Lange et al., 1998).

Scope of the thesis

The genus *Deinococcus* is rapidly expanding with a large majority of new species isolated by application of γ rays as a selective pressure. Deinococci have been the major focus of study with respect to the mechanism of its ionising radiation resistance. Also deinococci have been forwarded as the major candidate for bioremediation at the nuclear waste sites by its virtue to withstand with very high doses of ionising radiation. However such sites are often contaminated with other pollutants of particular importance are metal contaminants, which could have profound effect on deinococci.

The present study encompasses,

* The development of a molecular method based on 16S rRNA gene for the detection of deinococci from environment without using ionising radiation as selection pressure.

* The investigation of heavy metal tolerance of the radiation resistant bacteria and the mechanism of Cd^{2+} toxicity in *D. radiodurans* R1.

* Cloning and expression of synthetic metallothionein and a prokaryotic metallothionein, *smt*A in *D. radiodurans* R1.

Chapter 2: Development of a Deinococcus specific hemi-nested PCR method for the environmental detection and study of culture-independent diversity of Deinococcus spp.

When you have eliminated the impossible, whatever remains, however improbable, must be the truth.
- Sir Arthur Conan Doyle

2.1 Introduction

The family *Deinococcaceae* is represented by more than 40 validly described species, all (except one) belonging to a single genus, *Deinococcus* (Taxonomy Browser NCBI, *http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi),* which has members that are non-spore forming, gram positive, aerobic, chemorganotrophs, that exhibit remarkable resistance to radiation, desiccation and DNA damaging agents such as mitomycin (Battista, 1997; Cox and Battista., 2005; Blasisus et al., 2008; Slade and Radman, 2011). Study of this group of organisms is central for understanding the mechanism of radiation resistance (Battista, 1997; Liu et al., 2003; Daly et al., 2004; Daly et al., 2007). Their potential application in bioremediation of radioactive waste sites is also an important aspect (Daly, 2000; Fredrickson et al., 2000).

Environments from which deinococci have been isolated include diverse locations such as desert sand (de Groot et al, 2005, Rainey et al., 2005, Chanal et al., 2006), canned meats, (Anderson et al., 1956) geothermal springs (Ferreira et al., 1997), plant rhizospheres (Lai et al., 2006, Yang et al., 2008), and Antarctic soil (Hirsch et al., 2004). However the properties of these environments do not show any correlation that could proffer an explanation, based on natural selection, of their ionizing radiation (IR) resistant phenotype. It has been postulated that the IR resistance of *D. radiodurans* R1 (DR1) and its desiccation tolerance may be attributed to common machinery that repairs double-strand breaks in DNA (Slade and Radman, 2011).

Interest in the isolation of deinococcal cultures has led to the rapid expansion of the list of validly known species from only seven a decade ago (Battista, 1997) to about 47 in 2011 (Slade and Radman, 2011). Most often, for the isolation of deinococci, exposure of the total population to ionizing radiation is utilized as a means of enrichment (Ferreira et al, 1997; Rainey et al., 2005; Shukla et al., 2007), since the slow growth and fastidious nutrient requirements of deinococci makes them difficult to be isolated without eliminating the co-existing faster growing radiation–sensitive population. The utilization of strong selective conditions for their isolation, however, obliterates information about their relative abundance in different environments. As a result, despite their wide spread distribution in several niches, comprehensive study on deinococcal ecology is lacking.

In addition to deinococcal isolates, a number of environmental 16S rRNA gene sequences having deinococcal affinities are reported form different habitats (Ribosomal database Project *http://rdp.cme.msu.edu/index.jsp)*. These nucleotide sequences are mostly obtained by culture-independent approaches involving construction of rRNA gene libraries of the bacterial community present in the environment, followed by sequencing and identification of deinococcal clones from these libraries on the basis of their sequence similarity with the 16S rRNA gene sequences of identified isolates (Carpenter et al, 2000; Chanal et al, 2006). Based on the abundance of deinococcal clones in 16S rRNA gene libraries, it could be surmised that deinococci comprise a small fraction of the total bacterial community.

An alternative to total community rRNA gene libraries for culture independent study of bacterial populations is to selectively target the 16S rRNA gene sequences of a specific subpopulation, to study the composition and diversity of only that population from the environment. Such a study necessitates the development of unique DNA probes and/or group specific PCR primers for the selective study of that subpopulation. Such primers/probes may be species specific (Pulawska et al., 2006; Wong-Villarreal et al., 2010), genus specific (Jurado et al., 2006; Klocke et al.,2006; Kim et al., 2011), phylum specific (Blackwood et al., 2006; Muhling et al., 2008) or they could be specific towards a gene involved in a common functional attribute of that subpopulation (Táncsics et al., 2010; Dandie et al., 2007; Gomes et al., 2007; Antony et al., 2010). Group specific primers give better insight into the distribution, abundance and diversity of a specific sub-population. Of the several radiation resistant genera described *Rubrobacter* has been investigated using group specific primers (Holmes et al., 2000).

Wise et al., (1996) developed DNA probes based on the four deinococcal species described then. The designed probes were found to species specific. Peltola et al., (2008) have described 16S rRNA gene based PCR primers for quantification of *D. geothermalis*, so that they serve as species specific primers. Thus, PCR primers/DNA probes specifically and exclusively targeting majority if not entire deinococcal community are lacking and this precludes culture-independent studies addressing specifically the diversity and species composition deinococci.

Radiation resistant bacteria can serve as sensitive indicators of radiation pollution or excessive exposure to radiation. Development of group-specific primers for the detection of the members belonging to the genus *Deinococcus* will aid in easy and rapid detection and quantification of their distribution in several different environment that can aid in understanding of the evolution of the radiation resistance mechanism of the deinococci. This chapter deals with the development of 16S rRNA gene based PCR method for the rapid detection of deinococci from the environmental samples. The method is successfully applied to comprehend the diversity of deinococci by PCR-denaturing gradient gel electrophoresis (DGGE) and to establish the deinococcal identity of isolates obtained.

2.2 Materials and Methods

2.2.1 Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 2.1. The deinococcal cultures were grown in tryptone-glucose-yeast extract (TGY) medium (0.5 % tryptone, 0.1 % glucose and 0.3 % yeast extract) at 30 °C while non-deinococcal cultures were grown in Luria-Bertani (LB) medium at 30 °C except *E. coli* which was grown at 37 °C. *Bradyrhizobium japonicum* 61A152 was grown on Congo red-yeast extract-mannitol agar at 30 °C.

2.2.2 Bioinformatics tools used for the designing of Deinococcus specific primer

The 16S rRNA gene sequences of genus *Deinococcus* as listed in the Ribosomal Database Project (RDP) (*http://rdp.cme.msu.edu/*) were aligned using Clustal W (*http://www.ebi.ac.uk/clustalw*). The specificities of the conserved sequences were examined using the RDP PROBE MATCH tool by using sequences of approximately 20 nucleotide length as the query.

2.2.3 Soil sample collection

Soil samples from several arid and non-arid regions in India were collected. Top 5 cm layer of soil was collected and stored at 4 °C for culturable studies. An aliquot of the sample was preserved at -20 °C for community DNA isolation. The physico-chemical characteristics of the soil samples were determined at the Soil testing laboratory, Gujarat State Fertilizer Company (GSFC), Vadodara. The total Cr^{6+} concentration of chromium contaminated soil sample was estimated by heat digestion of soil sample by

HNO_3 + $HClO_4$ and analysed by Atomic absorption spectrophotometer (AAS) (GBC, Australia) at GSFC, Vadodara.

Table 2.1 Bacterial strains used in this study

Bacterial Strain	Description	Source or Reference
	Deinococcal strains	
D. radiodurans R1 ATCC 13939 (DR1)	Type strain	Prof. Mary Lidstrom, University of Washington, USA
D. radiophilus ATCC27603	Type strain	Dr. Y. N. Lee, Chungbuk National University, Korea
D. indicus MTCC 4913	Arsenic resistant	Microbial Type Culture Collection (MTCC), Chandigarh, India
Deinococcus sp. X2	Lab. isolate	Shukla et al., 2007
Deinococcus sp. G8	Lab. isolate	Shukla et al., 2007
	Non- Deinococcal strains	
Rhodococcus rhodochorous MTCC 2569	High G+C gram positive, Family *Nocardiacae*	MTCC, Chandigarh
Micrococcus flavus, NCIM 2763	High GC gram positive, Family *Micrococcacae*	National centre for industrial microorganisms, National Chemical Laboratory, Pune, India
Bacillus subtilis	Low GC gram positive, Phylum *Firmicutes*	Department culture collection
Bradyrhizobium japonicum 61A152	Phylum *Alpha-proteobacteria*	Joshi et al., 2008
Escherichia coli DH5α	Phylum *Gamma-proteobacteria*	Department culture collection, Sambrook and Russell, 2001

2.2.4 Isolation of radiation resistant bacteria

One gram of soil sample was irradiated for 10 kGy using Co_{60} source at4 kGy/h. The irradiated soil sample was suspended in 4.5 ml of saline (0.85 % NaCl), vortexed thoroughly and appropriate dilutions were plated on TGY agar plates and incubated for 4-5 days at 30 °C. Morphologically distinct colonies were purified and maintained on TGY plates.

2.2.5 Determination of UV tolerance of the bacterial isolates

The bacterial cultures were grown in TGY broth to late exponential phase, pelleted, washed with saline and OD_{600} adjusted to 0.4 and 10 ml of it was exposed to UV in a sterile petri plate using UV chamber (Wilber Lourmat, France) with 254 nm UV source. Aliquots of 1 ml were withdrawn at an interval of 200 J/m^2, appropriately diluted, and plated on TGY agar plates, incubated at 30 °C. The colonies were enumerated after 72 h.

2.2.6 Biochemical characterisation of the radiation resistant isolates

2.2.6 a Biochemical characters

Biochemical characterisation of the radiation resistant isolates was done according to the Bergey's Manual.

2.2.6 b Single carbon utilisation

Carbohydrate utilisation test was done using the Hi-Carbo test kit (Hi-media, Bombay, India). The bacterial cultures were grown to late exponential phase and washed with saline. A 50 µl of this was inoculated in each of the carbohydrate well provided in the kit and incubated at 30 °C for 48 h.

2.2.6 c Antibiotic –sensitivity test

A 100 µl of overnight grown culture was spread on TGY agar plates. The desired antibiotic octadisc was placed on the plate and incubated at 30 °C for 48 h.

2.2.6 d pH and temperature optima for growth of radiation-resistant bacterial isolates

To determine the temperature optima the cultures were grown in TGY and incubated at different temperatures. OD_{600} was monitored after 48 h. pH optima for the bacterial isolates was determined in TGY broth prepared in 0.1 M buffer of varying pH range. Acetate buffer for pH range 3, 4 and 5; phosphate buffer for pH 6 and 7; and Tris buffer for pH 8, 9, 10 were employed for determining the pH optima.

2.2.7 Soil community DNA isolation

Community DNA was isolated by the method described by Porteous et al., (1997). Briefly 0.5 g of soil was added to 925 µl of SDS lysis buffer (0.25 M NaCl, 0.1 M Na_2EDTA, 4 % SDS) and 75 µl of guanidine isothiocyanate, homogenized for 1-2 min by vortexing and then incubated for 1 h at 68 °C. The samples were then centrifuged at 13,000 g for 15 min at 4 °C and to the supernatant 300 µl of 30 % PEG

8000 in 1.5 M NaCl was added. The DNA was precipitated at -20 °C for 1 h followed by centrifugation at 13,000 g for 15 min at 4°C. Pellet was dissolved in 900 µl 2x CTAB solution (2 % hexadecyl trimethyl ammonium bromide, 1.4 M NaCl and 0.1 M Na_2EDTA) and incubated for 15 min at 68 °C. To the above solution 925 µl chloroform was added and gently mixed and centrifuged at 13,000 g for 10 min at room temperature. The aqueous DNA solution was precipitated with 1 ml isopropanol for at least 15 min at -20 °C, centrifuged at 13000 g for 15 min at 4 °C to collect the pellet which was dissolved in 450 µl 2.5M ammonium acetate (NH_4OAc) and 1ml of 95 % ethanol was added, kept for incubation at – 20 °C for 15 min and centrifuged at 13000 g for 15 min at 4 °C. The pellet obtained was then washed with 70 % ethanol and dissolved in minimum amount of double distilled water.

2.2.8 PCR amplification of 16S rRNA gene using universal primers

Universal eubacterial 16S rRNA PCR primers, Eub27F and Eub1107R (Table 2.2), obtained from MWG Biotech, Germany, were used for amplification of 16S rRNA gene from either pure cultures or from soil community DNA. PCR was carried out in 30 µl reaction mixture consisting of 1 ng of template DNA, 30 pmole of each of the primers, 1 µl of mixture of dNTPs 2.5 mM each, 1.5 U of *Taq.* DNA polymerase combined with appropriate amount of 10X *Taq.* DNA polymerase buffer. Amplification was carried out in a thermal cycler (Applied Biosystems, USA) with an initial denaturation at 94 ˚C for 3 min, followed by 30 cycles of denaturation at 94 ˚C for 45 s, annealing at 58 ˚C for 30 s, elongation at 72 ˚C for 1 min and 30 s, with a final elongation at 72 ˚C for 10 min. Amplicons were detected by electrophoresis on 1.0 % (w/v) agarose ethidium bromide gels.

2.2.9 PCR amplification of 16S rRNA gene using Deinococcus specific primer

16Sr RNA gene amplicon obtained as above (Section 2.2.8) was used as template for hemi-nested PCR using forward *Deinococcus* specific primer Deino202F (Table 2.2), (numbering corresponding to the 202-222 *D. indicus* 16S rDNA gene sequence) and 1107R as the reverse primer. Following additives were added to the PCR mixture individually or in combinations mentioned at a final concentration as given: betaine, 1M; DMSO, 10 %; glycerol, 10 % and formamide, 10 %. About 10- 50 ng of the template DNA was combined with 30 pmole of each of the primers, 1 µl of 2.5 mM

dNTPs, 1.5 U *Taq.* DNA polymerase in 10X *Taq.* DNA polymerase buffer. The PCR was carried out at an initial denaturation at 94 ˚C for 3 min followed by 30 cycles each consisting of denaturation at 94 ˚C for 45 s, annealing at 58 ˚C for 30 s, elongation at 72 ˚C for 1 min and 30 s repeated for 30 cycles with a final elongation at 72 ˚C for 10 min.

For amplification in presence of competing DNA, mixture of 20 ng each of 16S rRNA gene fragments amplified from different organisms was combined with varying concentrations of 16S rDNA of DR1 at final concentration and used as template for *Deinococcus* specific nested PCR.

Table 2.2 PCR primers used in this study

Primer	Sequence (5´-3´)	Reference
Eub27F	AGAGTTTGATCCTGGCTCCAG	Shukla et al., 2007
Eub1107R	GCTCG TTGCGGGACTTAACC	Shukla et al., 2007
Deino202F	GGGTTGCGTTCCATCAGC	This study
GC- P341F	CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG GCACGGGGGGCCTACGGGAGGCAGCAG	Muyzer et al., 1993
Eub 534R	ATTACCGCGGCTGCTGG	Muyzer et al., 1993

2.2.10 Construction and analysis of deinococcal 16S rRNA gene fragment library

The community rDNA gene fragments obtained by PCR amplification of soil community DNA using *Deinococcus* specific PCR was purified and cloned in the pTZ57R/T (InsTAclone, MBI Fermentas, USA) following the manufacturer's instructions and transformed in to *E. coli* DH5α. The presence of the recombinant plasmids in different transformants was confirmed by PCR with Deino202f and Eub1107R. The clones were analyzed by restriction fragment length polymorphism (RFLP) using restriction enzymes *Hha*I and *Msp*I. The digested products were electrophoresed using 8 % polyacrylamide gel and visualized by silver staining.

2.2.11 PCR-DGGE of 16S rRNA gene

An appropriate dilution of PCR product obtained upon *Deinococcus* specific hemi-nested PCR, using either environmental DNA or genomic DNA of a pure culture, was reamplified using GC- P341f, containing the GC clamp and Eub 534R by the protocol described by Muyzer et al., (1993). The products were analysed on 8 % polyacrylamide gel with denaturing gradient of 50 to 70 %, where 100 % denaturant contained 7 M urea and 40 % formamide. Electrophoresis was carried out using the D-code system (Bio-Rad, USA) for 16 h at 60 °C in 1X TAE buffer at 75 V, following which the DNA bands were visualized by silver staining. The DGGE gels were photographed and analysed by AlphaEase 4.0 software (Alpha Innotech , USA). The Shannon–Weaver index was calculated as

$$H= -\Sigma(n_i/N) \log (n_i/N)$$

Where n_i is the area of the peak and N is the sum of all the area covered under individual peaks of the densitometric curve. The DGGE bands were excised and gel pieces crushed in 50 µl distilled water, followed by centrifugation at 10,000 rpm for 3min. A 5µl aliquot of the supernatant was added to PCR reaction mixture for reamplification using the GC- P341f and Eub 534R primers. The reamplified bands were checked for their purity on DGGE gel as mentioned above and sequenced.

2.2.12 DNA sequencing and phylogenetic analysis

Cloned DNA samples were sequenced at Bangalore Genei Pvt. Ltd. (India). The phylogenetic analysis of the sequences was performed using the Tree Building tool at RDP (http://rdp.cme.msu.edu/). The BLASTn search tool (Altschul et al., 1990) was used to determine sequence homology and the most similar sequences in the GenBank database. All 16S rRNA gene sequences obtained in this study have been deposited in the GenBank and accession numbers are provided in the results section.

2.3 Results and Discussion

2.3.1 Designing and in-silico validation of Deinococcus specific primer

All the type strains representing genus *Deinococcus* listed at RDP (*http://rdp.cme.msu.edu*/) were used for the alignment using Clustal W and several patches of sequences conserved in all deinococci were obtained. The conserved sequences comprising of 18-mers were analysed using for their presence in other taxonomically distinct groups of bacteria. Table 2.3 lists the data for all the conserved

sequences and the hits in other phyla such as *Proteobacteria, Firmicutes* etc. The sequence corresponding to nucleotide positions 202-222 (numbered according to *D. indicus* sequence) had maximum coverage in the genus *Deinococcus.* This oligonucleotide sequence identified had 99.4 % hits in *Deinococcus*, demonstrated excellent match to this group (Table 2.3). Whether all deinococci show conservation of this sequence was ascertained and it was found that this sequence was present with 100 % match in 27 of the 40 species listed at the RDP database. The type strains *D. alpitudinis, D. claudioa and D. radiomolis,* were recognized with a single mismatch at 5′ end of the primer, indicating that these may be amplified by primers complementary to this sequence. Seven species viz. *D. misasensis, D. roseus, D. papagonenesis, D. periaridlitoris, D. sonorensis, D. maricopensis* and *D. pimensis,* form a coherent group in the *Deinococcus* clade not efficiently recognized by this primer. Earlier probes reported by Wise et al., (1996) as *Deinococcus* specific, when subjected to a similar analysis, were found to be present in maximum of 4 type strains of genus *Deinococcus*. Thus, an oligonucleotide primer corresponding to the 202-222 region, Deino202F (Table 2.2), was identified as a potential *Deinococcus* specific primer, targeting majority of species.

2.3.2 Development of Deinococcus specific hemi-nested PCR protocol

A two-step hemi-nested PCR approach was developed, in which the first amplification was done with universal eubacterial primers Eub27F and Eub1107R, followed by a second PCR, that utilizes the 16S rDNA form the first round as template, with *Deinococcus* specific primer, Deino 202F and universal eubacterial primer 1107R (Fig. 2.1).

The initial standardization of the protocol was done using genomic DNA as template, of DR1 as a positive control and of *E. coli* as a negative control. PCR reaction mixture containing 1M betaine was found to improve specificity at 58 °C, as opposed to other additives, to yield a band of expected band size, 900 bp, only with the deinococcal template DNA (Fig. 2.2). The GC content of the deinococci varies between 60-70 %; betaine, an isostabilizing agent, equalizes the contribution of GC- and AT-base pairing to the stability of the DNA duplex (Henke et al., 1997), possibly thereby improving the amplification. Therefore, subsequent PCR amplifications were carried out at annealing temperature of 58 °C in presence of 1 M betaine.

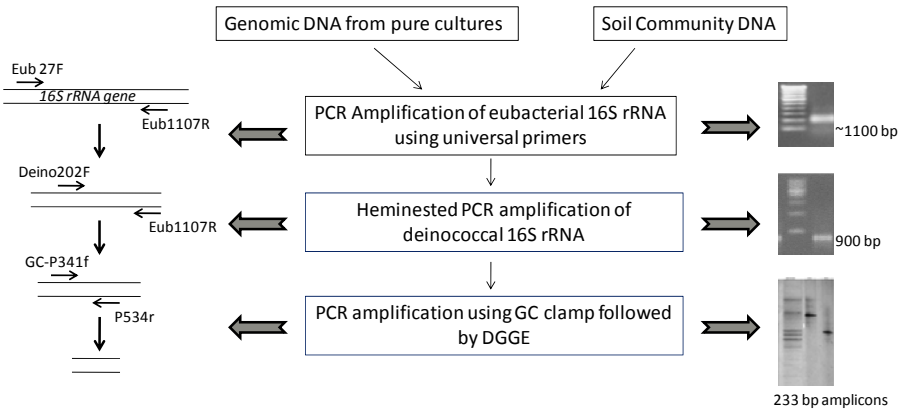


Fig. 2.1: Schematic representation of the *Deinococcus* specific heminested PCR protocol and culture-independent DGGE analysis of deinococcal diversity

Positive amplification was observed with *D. radiophilus* ATCC 27603, *D. indicus* MTCC 4913 and our laboratory isolates *Deinococcus* sp. X2 and *Deinococcus* sp. G8 (Shukla et al., 2007), but not with other phylogenetically diverse bacteria tested (Fig. 2.3).

For the suitability of the *Deinococcus* specific PCR to explore the diversity of this group from natural environments, the protocol should be robust in terms of its sensitivity so as to detect deinococci from mixed communities, in which deinococci are usually far out-numbered by other taxonomically distinct bacteria. An amplicon of 900 bp was obtained using 16S rDNA of *D. radiodurans* R1 ATCC 13939 with a sensitivity of 1 pg of first PCR product (Fig. 2.4a).

In order to study whether these primers are suitable for specific amplification of deinococcal 16S rRNA gene from a heterogeneous mixture containing template DNA from non-target organisms, a concoction of 20ng of each of 16S rDNA of the non-deinococcal representatives, *R. rhodochorous* MTCC 2569, *M. flavus, B. japonicum* 61A152, *E.coli* and *B. subtilis* was made and to this 16S rDNA of DR1 was added at different concentrations. This was used as a template for the second step of the *Deinococcus* specific nested PCR with Deino202F and 1107R as primers. An expected amplification of 900 bp was obtained in presence of competing non-target template DNA at all the concentrations (Fig. 2.4b).

Table 2.3: Specificity of the conserved regions of the aligned 16S rRNA genes of *Deinococcus* sp.

Region	Nucleotide position	Sequence	% Hits			
			Deinococcus	*Proteobacteria*	*Firmicutes*	Others
1	73-90	AGTGGCGCACGGGTGAGTA	1.166	66.66	0.84	31.260
2	202-222	GGGTTGCGTTCCATCAGC	99.4	0.5	0	0
3	265-285	CCGGCCTGAGAGGGTGGCCGG	33.8	6.08	12.5	47.52
4	288-308	ACAGGGGCACTGAGACACGGG	89.6	0.30	0	10.03
5fl	310-327	CCCACTCCTACGGGAGGC	61.062	0.871	0.435	37.63
5fr	328-345	AGCAGTTAGGAATCTTC	95.97	0.53	0.13	3.35
6	367-388	GAGCGACGCCGCGTGAGGGA	41.16	1.405	21.108	35.54
7fl	477-492	CGTGCCAGCAGCCGCG	0.209	35.008	28.36	36.41
7r	493-508	GTAATACGGAGGGTGC	0.86	81.53	0.04	90.09
8fl	510-529	AAGCGTTACCCGGAATCACTG	73.55	1.23	0.41	24.79
8fr	530-546	GGCGTAAAGGGCGTGTA	14.3	28.1	30.56	27.22
9fr	642-657	CTGGTGTAGCGGTGG	2.155	0.407	1.117	96.33
9fl	658-673	AATGCGTAGATACCAG	83.5	1.877	1.877	12.67
10	725-743	GCGCGAAAGTGTGGGGAGC	17.908	6.805	68.33	8.38
11fr	743-764	AACCGGATTAGATACCCGGG	19.46	0.38	3.8	76.34
11fl	765-785	TAGTCCACACCCTAAACGATG	65.14	15.22	0.07	18.37
12fr	839-857	CCGCCTGGGAAGTACGGCC	10.604	58.3	0.46	30.62
12fl	858-876	GCAAGGTTGAAACTCAAAG	0.521	17.96	65.86	15.65
13fr	878-899	AATTGACGGGGGCCCGCACAAG	0.345	37.37	21.651	59.37
13fl	900-922	CGGTGGAGCATGTGGTTTAATT	0.512	49.731	34.197	15.558
14	934-947	AAGAACCTTACCAG	1.13	15.71	65.64	17.50
15	983-997	GGTGCCCTTCGGGGA	3.81	29.42	64.70	2.050
16fr1	1012-1029	GCTGCATGGCTGTCGTCA	0.229	75.25	0.2921	24.22
16fr2	1030-1048	CTCGTGTCGTGAGATGTTG	0.339	37.59	37.85	24.21
16fl	1049-1076	GGTTAAGTCCCGCAACGAGCGCAACCC	0.338	36.988	35.085	27.58
17fl	1133-1154	GGAGGAAGGCGGGGATGACGTC	2.377	11.438	71.85	14.328
17fr	1155-1174	TAGTCAGCATGGTCCTTACG	98.63	0	0	1.36
18	1185-1201	ACACACGTGCTACAATG	0.44	46.667	35.56	17.31
19	1271-1286	AGTCTGCAACTCGACT	0.307	59.47	21.07	19.14
20	1296-1313	TGGAATCGCTAGTAATCG	0.34	29.64	44.05	25.95
21fl	1328-1349	GCGGTGAATACGTTCCCGGGCCT	0.461	30.214	25.480	43.843
21fr	1350-1373	TTGTACACACCGCCCGTCACACCA	0.151	57.56	32.244	10.041

fl and fr refer to the left and right half of the longer alignment blocks.

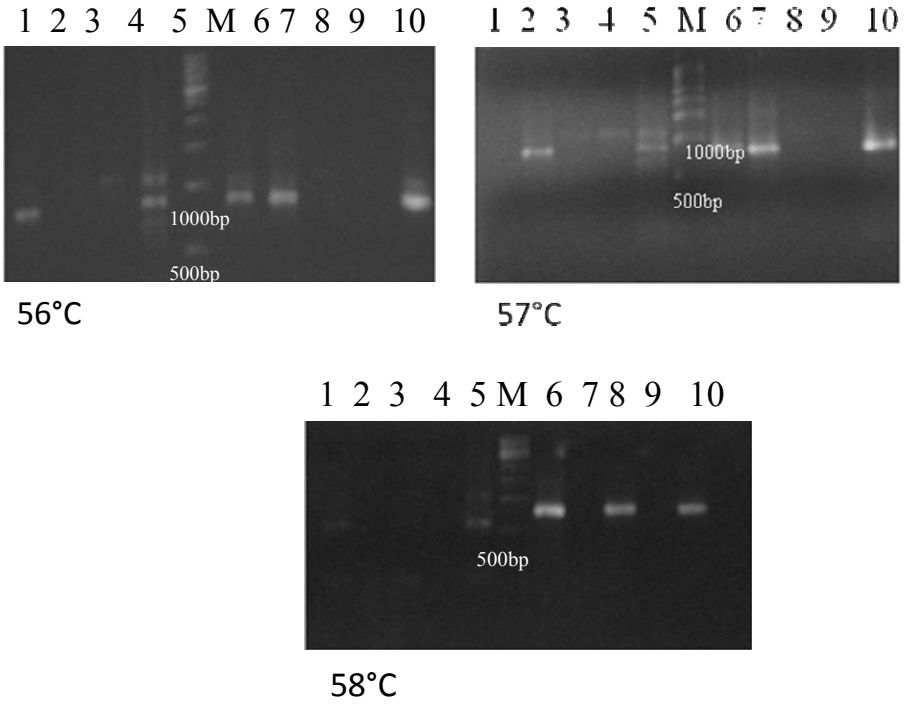


Fig. 2.2: Optimization of the *Deinococcus* specific hemi-nested PCR.16S rDNA was used as the template for the second round of nested PCR. Lanes 1-5 in each gel represent PCR products obtained using *E. coli* genomic DNA as template; lanes 6-10 with *D. radiodurans* R1 genomic DNA as template. Additives used for PCR are as follows: Lanes 1 and 6, 1M Betaine; Lanes 2 and 7, 2.5 M Betaine; Lanes 3 and 8, 10 % DMSO and 10 % Glycerol; Lanes 4 and 9, 10 % Formamide and 10 % Glycerol; Lanes 5 and 10, without any additive. M: (marker) 500 bp ladder. Annealing temperatures are given alongside the gel picture.

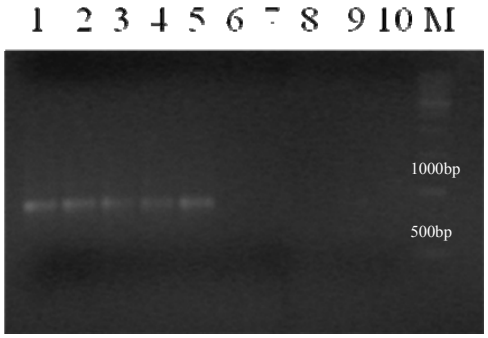


Fig. 2.3: Specificity of the *Deinococcus* specific hemi-nested PCR. Lanes : 1, *D. radiodurans* R1 ATTC13939; 2, *Deinococcus* sp.G8; 3, *D. indicus*;4, *Deinococcus* sp. X2; 5, *D. radiophilus*; 6, *R. rhodochorous* MTCC 2569; 7, *M. flavus*; 8, *B. japonicum* 61A152; 9, *E. coli*; 10, *B. subtilis*; Lane M, 500 bp ladder.

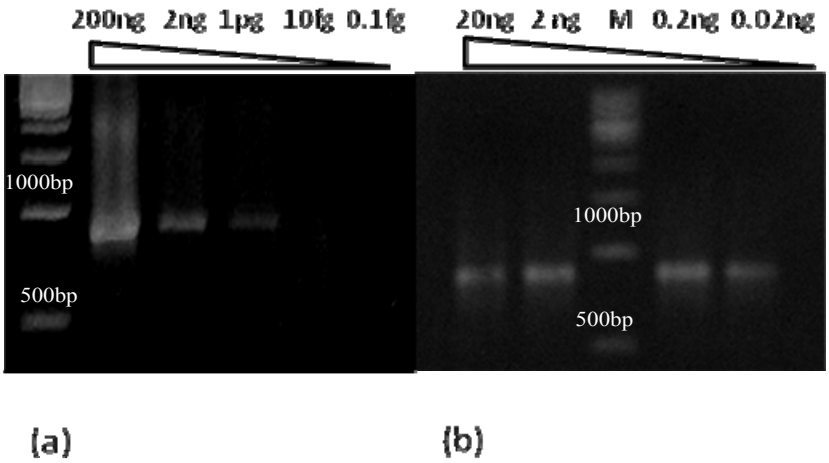


Fig. 2.4: Sensitivity of the hemi-nested PCR amplification for the amplification of deinococcal 16S rRNA gene fragments. a) Amplification using different amounts (as indicated) of DR1 16S rDNA as template. b) Amplification using mixture of 20 ng each of 16S rDNA from different pure cultures and spiked with varying concentrations of DR1 16S rDNA as indicated.

The sequence of the amplified product showed 100 % sequence similarity with DR1 16S rRNA gene. This signifies the specificity of designed primers in the detection of deinococci from a mixed population such as in soils.

2.3.3 PCR amplification of deinococcal 16S rRNA gene fragments from environment using Deinococcus specific primers

Samples were collected from western (GRK, LRK, CS, PP, CRC, BK), northern Himalayan samples (LD, LH, BR) and southern parts of India (CH) (Fig. 2.5). Sampling sites included diversified environments like salt deserts (LRK and GRK), cold arid environments (LD and LH) and contaminated soils (PP and CRC).The physico-chemical properties of the soil samples, as given in Table 2.4, show that samples varied in their physico-chemical properties, pH ranging from 7 to 8.5, while organic carbon ranged from 0.11-1.52 %. Soils with electrical conductivity (EC) > 4.0, such as samples LRK, CS, CH, are considered of high salinity (Yuan et al., 2007). The 16S rRNA gene was amplified from the total community DNA extracted from environmental samples and the resulting amplicon was subjected to hemi-nested PCR. Interestingly all the samples tested gave positive reaction however CS sample consistently gave a significantly smaller sized PCR product than expected (Fig. 2.6) indicating differences in this group. This infers the sensitivity of the designed *Deinococcus* specific PCR protocol to detect deinococci from diverse environments.

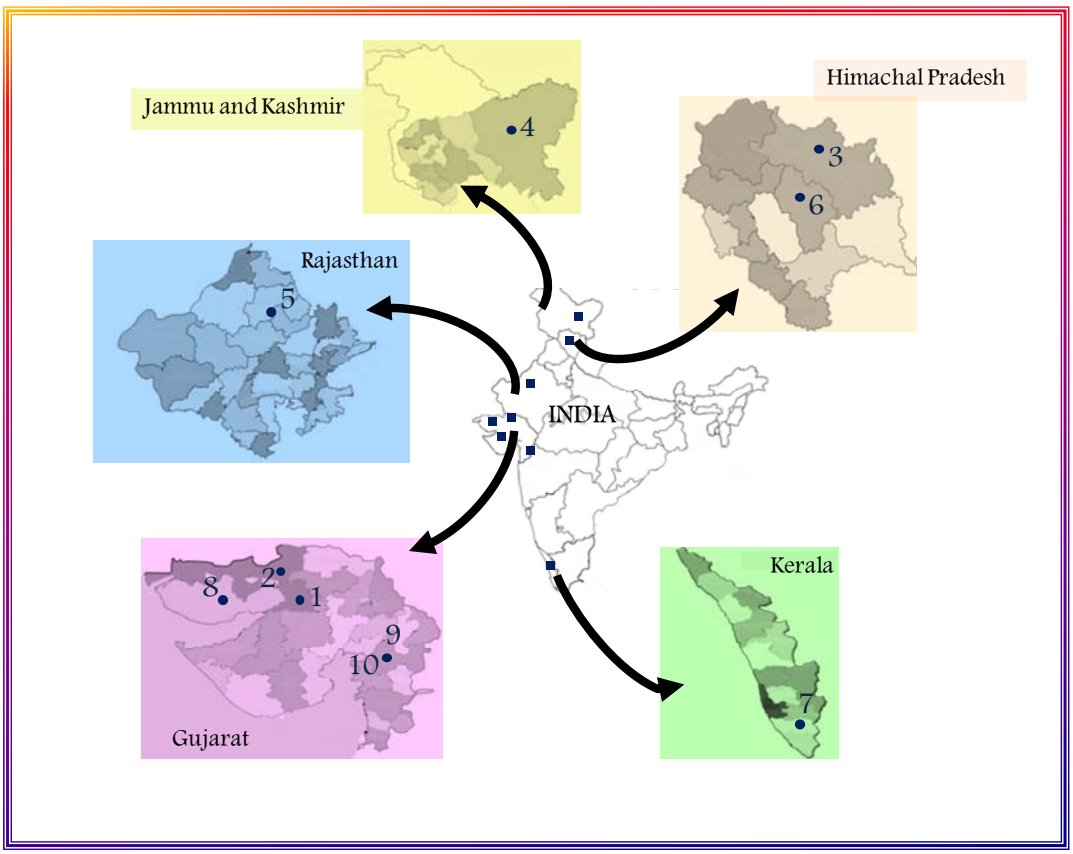


Fig. 2.5: Map showing the location of soil sampling sites. Central map shows locations of the sites along the country and the maps on the sides show more precisely the location within the states, with sample number given at the location in individual states. Samples locations are as follows with sample designations given in brackets. 1: Little Rann of Kutch (LRK); 2: Greater Rann of Kutch (GRK) 3: Lahaul (LH); 4: Ladakh (LD); 5: Bikaner (BK); 6: Soil at the banks of river Baes, Kullu (BR); 7: Chavra (CH); 8: Coastal soil (CS); 9: Petrol Pump (PP), Vadodara; 10: Chromium contaminated soil (CRC), Vadodara;

The ability to withstand high doses of radiation in bacteria is considered as an outcome of repeated cycles of hydration and desiccation despite the apparent absence of natural radioactivity on Earth (Cox and Battista, 2005; Shukla et al., 2007; Slade and Radman, 2011). Our results of positive amplification from xeric environments further affirm that such environments naturally select upon organisms that may be radiation resistant. Positive results from desert samples BK, LD, LH, GRK, is in coherence with the fact that deinococci have been routinely isolated from xeric environments such as the deserts of Sonoran (Rainey et al., 2005), Sahara (de Groot et al., 2005), Tataouine (Chanal et al., 2006), Kutch (Shukla et al., 2007), as well as cold deserts of Antarctica (Hirsh et al., 2004) and constitute about 37 % of all the type strains (Slade and Radman, 2011).

Table 2.4: Physico-chemical analysis of the soil samples

Sample Number	Location of soil sample collection site (Sample designation)	Sample collection period	pH	Organic Carbon (%)	Organic Nitrogen (%)	Electrical conductivity (1:2) $mScm^{-1}$
1.	Little Rann of Kutch, Gujarat (LRK)	Dec, 2007	7.45	0.50	0.043	17.31
2.	Greater Rann of Kutch, Gujarat (GRK)	Jan, 2007	8.15	1.30	0.1118	2.98
3.	Lahaul, Himachal Pradesh (LH)	July, 2006	7.13	1.43	0.122	0.22
4.	Ladhak, Jammu & Kashmir (LD)	June, 2006	8.23	1.25	0.1075	0.29
5.	Bikaner, Rajasthan (BK)	May, 2007	7.75	0.25	0.0215	1.08
6.	Beas River bank soil, Himachal Pradesh (BR)	May, 2006	7.15	0.14	0.0129	0.14
7.	Chavara, Kerala (CH)	Jan, 2007	8.55	0.11	0.00946	4.84
8.	Coastal soil, Kandla (CS)	Jan, 2005	8.37	1.25	0.1075	17.70
9.	Soil near petrol pump, Vadodara (PP)	Nov, 2006	6.97	1.52	0.1307	0.30
10.	Chromium contaminated soil, Vadodara (CRC)	May, 2007	8.30	0.82	0.070	2.32

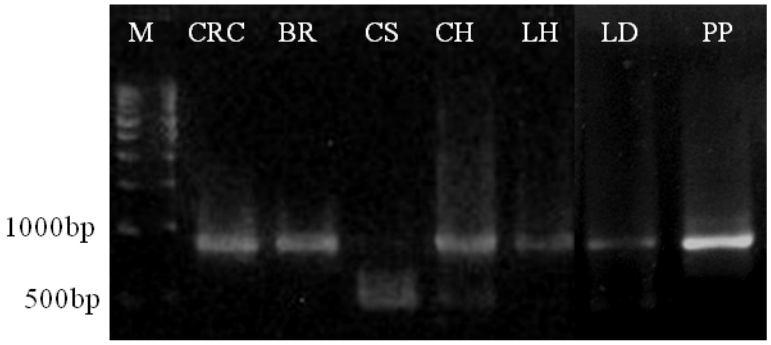


Fig. 2.6: *Deinococcus* specific nested PCR amplification from representative soil samples used in the study. M: 500 bp Marker. Sample names as given in Table 2.4.

Recently several deinococci have been reported from a radioactive spring samples (Asker et al., 2008; Wang et al., 2010; Peng et al., 2009; Asker et al., 2011). Chhavra (CH) lies in the south Indian state of Kerala, the coast of which contains the world's highest levels of natural radioactivity due to the local abundance of monazite, a mineral containing 10 % thorium phosphate (Forster et al., 2002). Detection of deinococci in CH sample emphasizes the enrichment of the deinococci due to high natural radioactivity. However deinococcal clones were not detected amongst 16S

rRNA gene library from uranium mining impacted soils in South Dakota, USA (Rastogi et al., 2010). Detection of deinococci from CRC sample, with chromium levels of 13.5 g kg^{-1} as determined by atomic absorption spectroscopy, as well as from petrol contaminated soil sample is interesting, since it is indicative of presence of strains possessing metal resistance or hydrocarbon tolerance, which are of significance in bioremediation of radioactive waste sites.

Two clone libraries using *Deinococcus* specific 16S rRNA gene were constructed from CRC and GRK samples. A total of 75 clones were obtained and Amplified Ribosomal DNA Restriction Analysis (ARDRA) revealed the presence of a single dominant clonal population in CRC sample, whereas GRK sample showed three different phylotypes. The sequences of four random clones, designated 6crc, 19crc, 22crc, and 25crc showed all of them to be affiliated to *D. radiopugnans* (Fig. 2.7) (Appendix II), suggesting the abundance of this species. *D. radiodurans* has been reported from vadose zone at the Hanford site having high Cr concentration as well as high background radioactivity. The Cr concentration at the Hanford site ranged from 0.02- 829.76 µg g^{-1} soil which is considerably lower than that found at CRC site (Fredrickson et al., 2004). The presence of deinococcal strains from polluted soils indicates the robust physiology of this group of organism.

GRK represents a salt desert habitat. Shukla et al., (2007) have earlier reported the isolation of deinococcal strain from such habitat however no attempt to study the uncultivable deinococcal diversity from such environments was made. This is the first attempt to assay the deinococcal diversity from such environment. Three different phylotypes were obtained from the clone library showing maximum homology to *D. hopeinsis*, and *D. murrayi* (Appendix II). Clone library from Calamita, black ferromagnetic sand from a marine iron ore on Elba Island (Italy) showed predominance of the clones that had affiliation to radiation resistant bacteria *Trupera* from the *Deinococcus –Thermus* decent and *Rubrobacter.* However no representation of deinococci was reported (Perfumo et al., 2011). *D. hopeinsis* has been earlier reported from Sonoran desert while there are no reports of isolation of *D. murrayi,* a moderate thermopile, from such environments.

2.3.4 Deinococcus diversity analysis using PCR-DGGE

To ascertain the suitability of the deinococcal nested PCR protocol for studying deinococcal diversity by a sensitive molecular fingerprinting technique, viz. PCR-

DGGE a three step PCR-DGGE was adopted wherein the nested PCR product was used as the template for amplification using GC- P341f and Eub 534r (Fig. 2.1). The protocol was adopted to enhance the representation of the *Deinococcus* specific sequences in the 16S rRNA gene pool of the community.

Various deinococcal pure cultures, when subjected to the three-step DGGE protocol, showed single bands at different positions, indicating 16S rRNA gene fragments showed sufficient sequence diversity to differentiate *Deinococcus* species. Environmental samples when subjected to three step deinococcal specific PCR-DGGE, showed several bands of varying intensities, with majority of samples having a single predominant band, suggesting dominance of a specific species as indicated by the clone library for CRC sample. Taking into consideration all the samples, about ten differently migrating major bands and several lighter bands could be discerned, demonstrating the high resolution of the method. It is known that culturable members of *Deinococcus* species isolated from similar environments are not necessarily phylogenetically closely related (Fig. 2.7) and our results are in coherence with the earlier observation.

Dar et al., (2005) have demonstrated that three step method increased the detection sensitivity of the sulphate reducing bacteria, otherwise a poorly represented population. The novelty of our approach is that the hitherto unknown diversity of major and minor representatives of deinococci was revealed as distinct ribotypes in the DGGE profile (Fig. 2.8 and Fig. 2.9). Fig. 2.9 demonstrates the relationship between the several sampling sites; the desiccated samples such as Ladakh and Little Rann of Kutch were related while those of Lahaul and Bikaner were related. Shannon–Weaver diversity index showed values ranging from 0.64 to 3.01 (Table 2.5), indicating variation in the species abundance. The higher indices of samples from salt desert of Gujarat (GRK and LRK) are suggestive of high species richness. The identity of the DGGE bands marked in Fig. 2.8, as revealed by their nucleotide sequences, is provided in Table 2.6. The sequence of the major band in CRC sample is in congruence with the observation from CRC group specific clone library. 16S rRNA gene sequences similar to *D. radiopugnans* were represented from diverse environments such as coastal region of Chhavra and banks of Baes river. Several *D. radiopugnans* have been demonstrated as a predominant member in river soil samples from Nottingham, UK (Masters et al., 1991). Ladakh is a region belonging to the northern Greater Himalayas characterized by extreme environments and low

precipitation. 16S rRNA based studies embarked to elucidate the diversity of regions of Himalaya do not show representation of deinococci (Pradhan et al., 2010). Our protocol was able to detect deinococci from this sample and sequences showing maximum similarity to *D. deserti*, a strain earlier isolated from Sahara desert (de Groot et al., 2005). Sequences from CS (8B3) and PP (9B1) showed maximum identity to *D. proteolyticus,* and *D. radiodurans,* respectively. The 16S rRNA gene library from cyanobacterial mats at the petroleum contaminated at the Arabian Gulf coast was shown to have clones of deinococcal affiliation (Abed et al., 2006). *D. murrayi*, represented diversity of GRK. Nevertheless none of these isolates, showing best match with the DGGE bands, have been described from any other region expect from their initial source of isolation.

2.3.5 Culturable diversity of the radiation resistant bacteria

In an effort to isolate ionising radiation bacteria from the soils described in the study, the samples from different habitats were subjected to γ radiation and the surviving organisms were plated. *Deinococcus* sp. are known to tolerant to both ionizing as well as non-ionizing radiation, therefore, UV radiation resistance of the isolates was studied (Table 2.7). Of the 25 isolates obtained from 5 samples, isolates Grk4, Grk5, Lrk4, Crc4, PP2, PP3 could tolerate up to 1000J/m^2 while Grk2, Crc1, PP1 showed an appreciable resistance of up to 800 J/m^2 (Table 2.7). The hemi-nested approach was employed to ascertain whether the isolates belonged to the genus *Deinococcus*. Interestingly, only four of the UV resistant isolates i.e. three isolates of GRK sample, Grk2, Grk4 and Grk5, while one isolate of LRK sample, isolate Lrk4 could be assigned to the *Deinococcus* as they gave positive amplification in hemi-nested PCR approach. The other isolates may not be deinococci but may belong to other eubacteria genera that have been reported to be radiation resistant such as the *Rubrobacter*, *Kinecococcus*, or members of genus *Methylobacteria*, *Lactobacillus* (Cox and Battista, 2005). Alternatively they may belong to the deinococcal clade which is not recognized by the specific primer Deino202f (Fig. 2.7). The UV resistance of the GRK isolates (Fig. 2.10) is comparable to the type strain DR1. Grk4, Grk5 are more resistant to UV while Grk2 is sensitive than type strain DR1. Partial sequence of Grk2, Grk4, Grk5 confirmed them to be *D. proteolyticus* and *D. ficus* respectively Table 2.8 and Fig. 2.7.

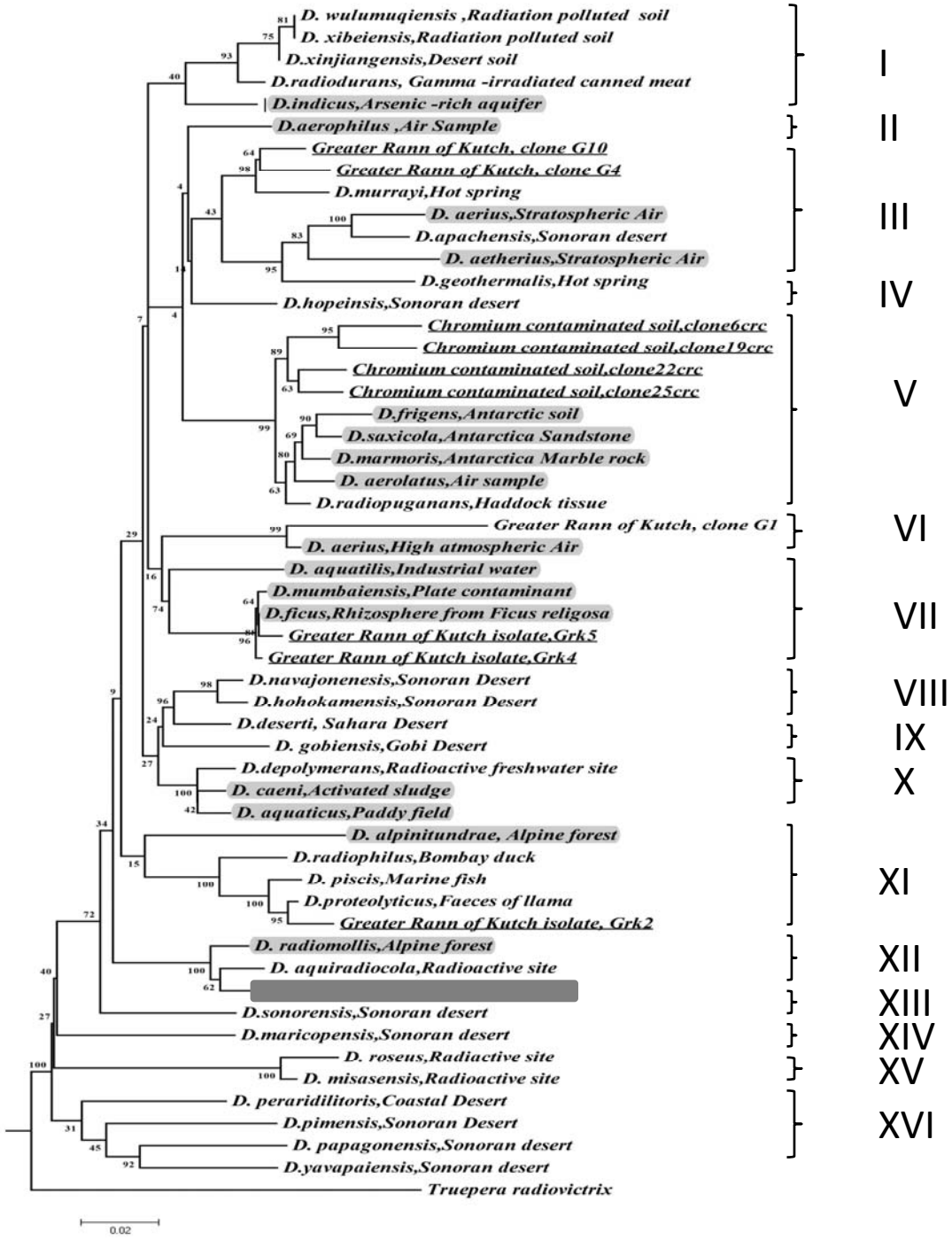


Fig. 2.7: Neighbor-joining tree based on 16S rRNA gene sequences showing the phylogenetic affiliation with type strains of *Deinococcus* species as listed in RDP. *Trupera radiovitrix* is used as outgroup; The cloned sequences from the CRC, GRK sample and the isolates from Grk2 are underlined. Bootstrap values based on 100 replicates are shown near the branch points. The bar indicates the Jukes–Cantor distance of 0.02. Information regarding the environmental niche from where the strains were isolated has been added against the strain name and highlighted isolates are the strains that were isolated from samples that had no exposure to radiation treatment prior to isolation.

Table 2.5: Diversity indices for deinococcal community from different soil samples

Sample	LRK	GRK	LD	LH	BK	BR	CH	MS	PP	CRC
Shannon Weaver index (H′)[a]	1.861	2.233	3.019	2.494	2.702	0.951	2.156	2.451	2.104	0.604
Evenness (E)	0.895	0.970	0.976	0.920	0.935	0.866	0.936	0.986	0.957	0.871
Simpson's Dominance (D)	0.152	0.112	0.059	0.102	0.077	0.432	0.132	0.101	0.132	0.585

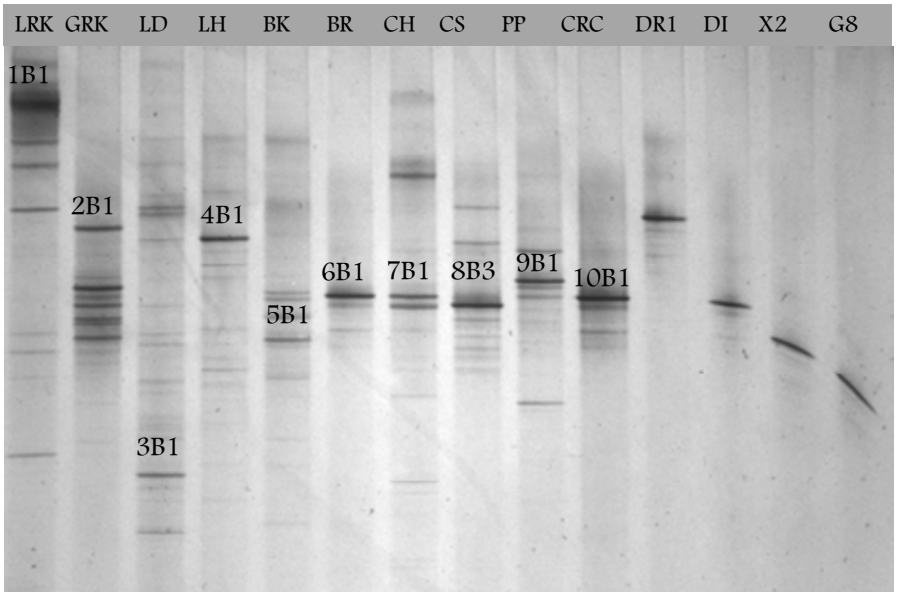


Fig. 2.8: PCR-DGGE analysis of deinococcal diversity from environmental samples.R1, DI, X2, G8 are control samples using pure cultures of *D. radiodurans* R1, *D. indicus, Deinococcus* sp. X2 and *Deinococcus* sp. G8. Lanes with environmental samples are named according to the soil sample designations are as given in Table 2.4.

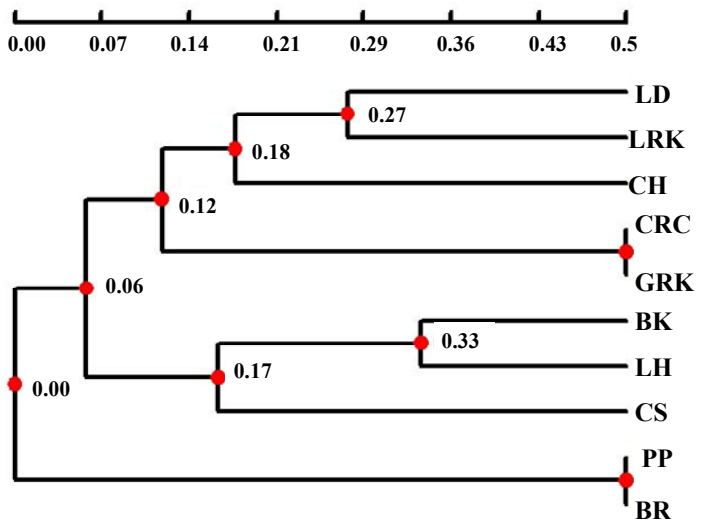


Fig. 2.9: Dendrogram for the DGGE profile from soil bacterial communities. UPGMA dendrogram was constructed using Dice's similarity index. The scale indicates similarity amongst the soil samples.

Table 2.6: Sequence similarity of representative bands excised from the DGGE gel shown in Fig. 2.8 (Appendix I).

Sample	DGGE band	Genbank accession No.	Best Match (Genbank accession No.)	% similarity
GRK	2B1	FJ548964	*D. murrayi* DSM11303 (NR026416)	96
LD	4B1	FJ548965	*D. deserti* VCD115 (NR043243.1)	98
BR	6B1	FJ548966	*D. radiopugnans* ATCC19172 (NR026403.1)	97
CH	7B1	FJ548967	*D. radiopugnans* ATCC19172 (NR026403.1)	97
CS	8B3	FJ548968	*D. proteolyticus* MRP (NR026400.1)	95
PP	9B2	FJ548969	*D. radiodurans* DSM20539 (NR026401.1)	95
CRC	10B1	FJ548970	*D. radiopugnans* ATCC19172 (NR026403.1)	97

Table 2.7: Characterization of gamma radiation enriched colonies for UV resistance and PCR with *Deinococcus* specific hemi-nested protocol

Sample	Isolate	UV resistance	*Deinococcus* specific hemi-nested PCR
Greater Rann of Kutch (GRK)	Grk1	-	-
	Grk2	+	+
	Grk3	-	-
	Grk4	++	+
	Grk5	++	+
Little Ran of Kutch (LRK)	Lrk1	-	-
	Lrk2	-	-
	Lrk3	-	-
	Lrk4	++	+
Chromium Contaminated Soil (CRC)	Crc1	+	-
	Crc2	-	-
	Crc3	-	-
	Crc4	++	-
	Crc5	-	-
Petrol Pump soil (PP)	PP1	+	-
	PP2	++	-
	PP3	++	-
	PP4	-	-
	PP5	-	-
Bikaner Soil (BK)	BK1	+	-
	BK2	-	-
	BK3	-	-
	BK4	-	-
	BK5	-	-

+ Tolerates up to 800J/m^2; ++ Tolerates up to 1000J/m^2.

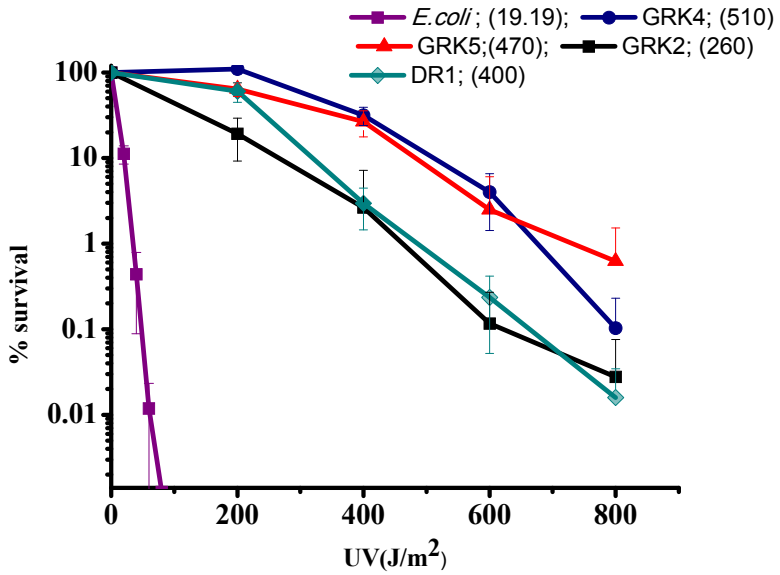


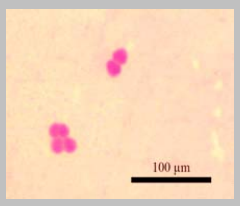
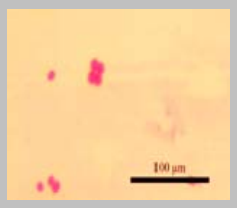
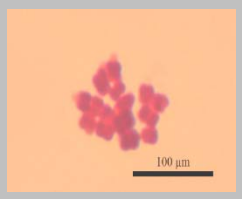
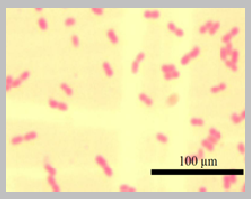
Fig. 2.10: UV tolerance of the radiation–resistant bacterial isolates. Values in parentheses against the strain names are D_{10} values, i.e. the UV dose required for 90 % killing, in J/m^2.

Table 2.8: 16S rRNA gene sequence match of the radiation resistant isolates (Appendix III)

Isolate	GenBank Accession No.	Best Match (Genbank Accession Number)	% similarity
Grk2	HQ738629	*D. proteolyticus* MRP (NR_026400.1)	98
Grk4	HQ876598	*D. ficus* CC-FR2-10 (NR_043282.1)	98
Grk5	HQ738630	*D. ficus* CC-FR2-10 (NR_043282.1)	93

The biochemical test that characterize genus *Deinococcus* show that all the three isolates could grow on 1 % NaCl, were catalase positive and could reduce nitrate. Grk2 and Grk5 could hydrolyse esculin while Grk2 like the standard strain DR1 could not hydrolyse esculin (Table 2.9). The isolates Grk2, Grk4, Grk5 were compared for carbohydrate utilisation (Table 2.10) and antibiotic resistance (Table 2.11).

Table 2.9 Morphological and biochemical characteristics of the radiation resistant bacterial isolates.

Characteristics	DR1[a]	Grk2	Grk4	Grk5
Arrangement of cells	Gram positive, Cocci[†] 	Gram positive, Cocci[†] 	Gram positive, Cocci[†] 	Gram positive, Cocci[†] 
Motility	-	-	-	-
Growth with 1% NaCl	+	+++	+++	+++
NO_3^- reduction	+	+	+	+
Esculin hydrolysis	-	++	-	++
ONPG[b] hydrolysis	-	-	-	-
Catalase	++	++	++	++

[a] DR1 refers to *D. radiodurans* R1

[b]ONPG ortho-nitrophenyl-β-D-galactoside

[†] Gram- stained cells and bar each of the micrograph denotes 100 µm.

Grk2 and Grk5 showed similar carbohydrate utilization. All the isolates could utilize fructose as the standard strain DR1. The isolates have broader carbohydrate utilization spectrum as opposed to the standard strain DR1 indicating biochemical versatility.

The strain Grk4 was sensitive to all the antibiotics tested while Grk5 and Grk2 were resistant to colistin. Grk2 also showed resistance to oflaxcin. From the comparison of the biochemical, carbohydrate utilization and antibiotic resistance the isolates Grk2 and Grk5 were demonstrated to be similar.

Table 2.10: Carbohydrate utilisation of the radiation resistant bacterial isolates

Carbohydrate	DR1	Grk2	Grk4	Grk5
Lactose	-	-	-	-
Xylose	-	-	+	-
Maltose	-	-	-	-
Fructose	++	++	++	++
Dextrose	+	++	++	++
Galactose	-	-	-	-
Raffinose	+	-	-	-
Trehalose	+	-	+	-
Melibiose	-	-	-	-
Sucrose	+	++	++	-
L-Arabinose	-	-	-	-
Mannose	+	++	+	++
Inulin	-	++	++	-
Sodium gluconate	+	-	-	-
Glycerol	-	-	-	-
Salicin	-	+	-	-
Glucosamine	-	-	-	-
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Sorbitol	-	+	+	-
Mannitol	-	-	-	-
Adonitol	-	-	-	-
α-methyl-D-glucoside	+	+	-	-
Ribose	-	+	+	-
Rhamnose	-	+	++	++
Cellobiose	-	-	-	++
Melezitose	-	++	-	++
α-methyl-D-mannoside	-	++	-	++
Xylitol	-	++	-	++
ONPG	-	-	-	-
Esculin	-	++	-	++
D-Arabinose	-	-	-	-
Citrate	-	-	-	-
Malonate	-	-	-	-
Sorbose	-	+	-	-

Table 2.11: Antibiotic susceptibility of the radiation resistant bacterial isolates

Antibiotic	Grk2	Grk4	Grk5
Cephalothin (Ch) 30µg	S(--)	S(-)	S(--)
Clindamycin (Cd) 2 µg	S(--)	S(--)	S(--)
Co-Trimoxazole(Co) 25 µg	S(-)	S(--)	S(--)
Erythromycin (E) 15 µg	S(--)	S(--)	S(--)
Gentamycin (G) 10 µg	S(-)	S(-)	S(-)
Oflxacin (Of) 1 µg	R	S(-)	S(--)
Penicillin G (P) 10U	S(--)	S(--)	S(--)
Vancomycin (Va) 30 µg	S(--)	S(-)	S(--)
Ampicillin (A) 25 µg	S(-)	S(--)	S(--)
Tetracycline (T) 10 µg	S(-)	S(--)	S(-)
Cephradine (Cv) 30 µg	S(-)	S(--)	S(--)
Carbenicillin (Cb) 100 µg	S(--)	S(--)	S(--)
Colistin (Cl) 50 µg	R	S(-)	R
Ceftriaxone (Ci) 30 µg	S(--)	S(--)	S(--)

The isolates Grk2 and Grk4 could grow over a broader range of pH, ranging from pH 5-9, while Grk5 grew well between the pH 5-7 (data not shown). The temperature optima of all the isolates were in the range of 30-40 °C (Fig. 2.11).

2.4 Conclusion

Majority of studies characterizing deinococci from environments involve enrichment of samples by a high dose of radiation, a step which obliterates information about species abundance and diversity and gives no clue about the uncultivable deinococci in the environment.

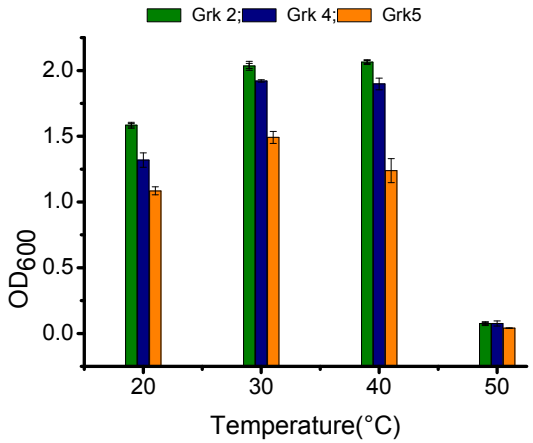


Fig. 2.11: Growth of radiation resistant bacterial isolates at different temperatures.

This work presents for the first time a comprehensive study of the diversity of deinococci by using culture independent approach by a newly developed hemi-nested 16S rRNA gene based PCR protocol. The application of this protocol was demonstrated (i) for ascertaining presence of deinococci from several diverse environmental samples, as they are rare members of natural communities; (ii) for confirmation of radiation resistant isolates as deinococci by a simple PCR tool; (iii) for studying deinococcal diversity by using multiple approaches such as clone library construction and fingerprinting technique such as DGGE; and (iv) for obtaining valuable information about species richness and abundance, since enrichment and isolation of bacteria by radiation treatment is not necessary for detection and quantification of deinococci by this method. The protocol developed has the advantage that it is sensitive enough to detect deinococci directly from community DNA samples without exposing the native population to the strong selective pressure imposed by radiation. In an interesting approach adopted by Suen et al., (2007) of using genome sequences to define ecological niche, the authors have placed *Deinococcus* species in the same niche along with several phylogenetically unrelated members, many of which show radiation resistance and xeric tolerance. The protocol developed in the current study will help experimentally confirm the predictions of these authors, since many environments can be easily sampled. It should also be possible to use the hemi-nested PCR approach for *in situ* hybridization and for quantitative PCR.

Chapter 3: Heavy metal interactions in <u>Deinococcus</u> spp.

A scientist in his laboratory is not a mere technician: he is also a child confronting natural phenomena that impress him as though they were fairy tales – Marie Curie

3.1 Introduction

The production of energy from nuclear power plants, uranium mining, nuclear weapons production and nuclear accidents are the major causes of release of radionuclides into the environment. The nuclear wastes typically contain inorganic and organic contaminants that include radionuclides, heavy metals, acids/bases and solvents. The nuclear wastes are pre-dominantly contaminated with radionuclides such as uranium, plutonium, caesium, organo-pollutants (e.g. toluene, benzene, ethylbenzene, xylene etc.), and heavy metals (lead, mercury, chromium, arsenic and cadmium) (NABIR primer 3;*http://www.lbl.gov/NABIR*; Daly, 2000). The high radiation levels, in combination with the chemical hazards, causes extreme damage to ecosystem and living organisms.

The clean-up of nuclear waste by physico-chemical methods is impractical and the cost is prohibitive. A less expensive *in situ* bioremediation technology is being investigated as a potential alternative method for treating such contaminated sites. Generally, bacteria used for bioremediation are selected to target a specific form and oxidation state of toxic pollutants, such as reduction of soluble hexavalent uranium or degradation of a specific hydrocarbon. However, since radioactive waste sites are rarely contaminated by a single chemical, it is necessary for the bioremediating strain to be multi-resistant to various toxic agents. These vast waste sites are therefore potential targets for utilizing specialized microorganisms that have the ability to survive and to catalyze the desired function(s) under radiation stress.

The development of bioremediation strategies using *Deinococcus* sp., the members which are among the most radiation resistant organisms known, are therefore vital for the clean-up of radioactive waste sites. Additional advantages of deinococci are that they are vegetative, easily cultured and non-pathogenic. Due to the common presence of toxic heavy metals in waste sites, there exists a considerable interest in studying physiology and the genes involved in metal resistance and reduction for the common metallic waste constituents.

D. radiodurans can naturally reduce Cr^{6+} to less mobile and less toxic form Cr^{3+} (Fredrickson et al., 2000). Additionally, genes from other organisms are being expressed in deinococci to impart resistance as well as develop ability to transform those metals. Brim et al., (2000) have generated *D. radiodurans* strains expressing the cloned Hg^{2+} resistance gene (*merA*) from *Escherichia coli* BL308. *MerA* encodes mercuric ion reductase, which reduces highly toxic, thiol-reactive mercuric ion, Hg^{2+},

to less toxic and inert elemental and volatile Hg^{o}. The strains were shown to grow in the presence of both radiation and ionic mercury at concentrations well above those found in radioactive waste sites, and to effectively reduce Hg^{2+} to less toxic volatile elemental mercury.

Metal interactions in *D. radiodurans* have been of interest not only from the point of view of metal detoxification/remediation but also due to the important role that certain heavy metals play in the radiation resistance physiology of this organism. *D. radiodurans* accumulates exceptionally high intracellular manganese and low iron levels. Accumulation of Mn^{2+} in deinococci is important for radiation resistant phenotype by facilitating recovery from radiation damage (Daly et al., 2004). It is believed that Fe-rich, Mn-poor cells are killed rapidly even at low radiation doses possibly due to the release of Fe^{2+} from proteins during irradiation, leading to cellular damage by Fe^{2+}-dependent oxidative stress. In contrast, Mn^{2+} ions concentrated in *D. radiodurans* might serve as antioxidants that reinforce enzymic systems which defend against oxidative stress during recovery (Ghosal et al., 2005).

Cadmium, an element with virtually no biological function, is a highly toxic metal. Cd^{2+} ions mediate their toxic effects by the induction of oxidative stress and also due to their strong affinity for –SH groups and ability to compete with other divalent metal ions for binding to proteins. The effect of this metal on radiation resistance in deinococci has not been well explored. The present chapter deals with the comparison of heavy metal resistance/tolerance of *D. radiodurans* R1 (DR1) and other newly isolated strains of deinococci. A detailed study of effect of Cd^{2+} in relation to Mn^{2+} in various physiological responses is presented.

3.2 Materials and methods

3.2.1 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are described in Table 3.1. The deinococcal cultures were grown in TGY (0.5% tryptone; 0.1% glucose; 0.3% yeast extract). The *D. radiodurans* R1 (DR1) mutants were cultured on kanamycin (50 µg/ml), chloramphenicol (3 µg/ml), or hygromycin (25 µg/ml) as required. *E.coli* DH5α cultures were grown at 37°C in Luria–Bertani (LB) broth or on LB agar. As per requirement *E.coli* DH5α was grown in L.B. supplemented with ampicillin at a final concentration of 100µg/ml (pTZ7R/T) and 50 µg/ml (pRADZ3).DR1 carrying

pRADZ3 and its derivative were grown in TGY supplemented with 3 µg/ml chloramphenicol.

Table 3.1 Bacterial strains and plasmids used in the study.

Bacterial strain	Relevant information	Source/Reference
E.coli DH5α	*supE44* D*lacU(f80lacZDM15) hsdR17 recA1 endA1 gyrA96 thi 1 relA1*	Laboratory stock
D. radiodurans R1 ATCC13939	Wild type	Prof. Mary Lidstrom, University of Washington, U.S.A.
Deinococcus sp. Grk2	Isolate from Greater Rann of Kutch, Gujarat, India.	This study, Chapter 2
*Deinococcus sp.*Grk4	Isolate from Greater Rann of Kutch, Gujarat, India.	This study, Chapter 2
Deinococcus sp. Grk5	Isolate from Greater Rann of Kutch, Gujarat, India.	This study, Chapter 2
D. radiodurans MD885, *sodA*$^-$	R1 but *sod A:: aph*	Prof. M. J. Daly, Uniformed Services, University of the Health Sciences, Bethesda, U.S.A. Markillie et al., (1999)
D. radiodurans JAK1, *pprI*$^-$	R1 but *pprI536::aph*	Prof. I. Narumi, Japan Atomic Energy Agency. Ohba et al. (2005)
D. radiodurans XCSP1, *pprM*$^-$	R1 but *pprM286::hph*	Prof. I. Narumi, Japan Atomic Energy Agency, Ohba et al., (2009)
D. radiodurans TNK 106, *recA*$^-$	R1 but *recA::cat*	Prof. John Battista, Louisiana State University Tanaka et al., (2004)
	Plasmids	
pRADZ3	Shuttle vector between *E.coli* and *D.radiodurans* R1, *groESL* promoter:: *lacZ* fragment of pMUTIN2mcs;10kb;*E.coli*(Amp^r) and Cat^r (DR1)	Prof. Mary Lidstrom, University of Washington, U.S.A. Meima and Lidstrom, (2000)
pTZR/T-p*rec*A	T-vector containing *rec*A promoter; Amp^r	This study
pRADZ3-p*rec*A	pRADZ3 with P*rec*A *::lacZ* reporter	This study

Aph: Kanamycin resistance; hph: Hygromycin resistance; *cat*: chloramphenicol

3.2.2 DNA manipulations

Mini scale plasmid isolation from *E.coli,* restriction digestion and ligation were performed by the general protocols described by Sambrook and Russell, (2001). All the restriction enzymes were either procured from Bangalore genei Pvt. Ltd. (Bangalore, India) or MBI Fermentas (Germany). DR1 was transformed by the $CaCl_2$ method described by Satoh et al., (2009). Briefly, DR1 cells (1 ml) grown to early stationary phase (16 h of approx. 1.2 $OD_{600\ nm}$) were harvested by centrifugation, at 9650 g for 3 min., washed with 1 ml of TGY broth, resuspended in 0.1 ml of TGY broth, amended with 40 µl of 0.3 M $CaCl_2$. A 30 µl aliquot of the cell mixture and 10 µl of plasmid DNA (200–400 µg/µl) were mixed in a new culture tube and incubated at 30 °C for 90 min. To this 2 ml of TGY broth was added and the mixture was incubated at 30 °C for 24 h. The culture was appropriately diluted with 10 mM sodium phosphate buffer (pH 7.0) and spreaded on TGY plates supplemented with 3 µg /ml chloramphenicol. The transformants were scored after 3-5 days of incubation at 30 °C.

3.2.3 Influence of heavy metals on growth of radiation resistant bacteria

The heavy metal tolerance of the cultures was determined in TGY broth using a late logarithmic phase culture. TGY containing tubes were amended with different metals ($CdCl_2$, $HgCl_2$ and $K_2Cr_2O_7$) at concentrations mentioned and were inoculated at an initial OD_{600} of 0.1. The cultures were incubated at 30 °C under shaking condition for 12 h after which growth was recorded as OD_{600} measurements and expressed as percentage survival by considering the growth in media without any metal as 100 %. To study the growth phase dependence on metal tolerance, the cultures used for inoculum were grown to either late log phase, 12 h, or stationary phase, 48 h, inoculated at an initial OD_{600} of 0.1. To analyse the effect of Mn^{2+} on Cd^{2+} toxicity, 100 µM of Mn^{2+} was added to the media. Percentage survival was calculated as described.

3.2.4 Influence of Cd^{2+} and Mn^{2+} on recovery from H_2O_2 damage

Exponentially growing bacterial cultures were freshly inoculated in TGY broth at an OD_{600} of 0.1. Cultures were allowed to grow for 8 h and the OD_{600} was set to 0.6. H_2O_2 was added at a concentration of 20 mM. Aliquots were taken at different time intervals and appropriate dilutions were plated on TGY and TGY amended with 2.5 µM Cd^{2+} or 100 µM Mn^{2+}. Surviving fraction was enumerated after incubation for 48 h at 30 ˚C.

3.2.5 Influence of Cd^{2+} and Mn^{2+} on recovery from UV damage

The deinococcal cultures were grown to late exponential phase, washed with 0.8 % saline and OD_{600} adjusted to 0.4. The suspension (10 ml) was taken in sterile petri plate and irradiated in UV chamber with 254 nm UV source (Wilber Lourmat, France). Aliquots were withdrawn after exposing to UV doses of an increment of 200 J/m^2 and appropriate dilutions were plated on TGY and TGY amended with 2.5 µM Cd^{2+} or 100 µM Mn^{2+}. The colonies were enumerated after 72 h at 30 ˚C.

3.2.6 Determination of reactive oxygen species (ROS) in bacterial cells

Cells grown aerobically in TGY medium to an OD_{600} ~0.4, were exposed for 90 min to varying concentration of Cd^{2+} and H_2O_2. Cell suspensions were centrifuged, washed with 10 mM potassium phosphate buffer, pH 7.0, and incubated for 45 min in the same buffer containing 10 µM 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), dissolved in dimethyl sulfoxide. Cells were subsequently washed and resuspended in 250 µl of 10 mM potassium phosphate buffer, pH 7.0 and disrupted by sonication (Perez et al., 2007). One hundred microliters of the resulting cell extracts were mixed with 1 ml of the same buffer and fluorescence intensity was determined using Thermo-Spectronic, Model F-7000 FL Spectrophotometer (excitation 490 nm, emission 519 nm). Emission values were normalized to protein concentration determined by Bradford's method (Bradford, 1976).

3.2.7 Assay of reactive oxygen species (ROS) combating enzymes

a) Superoxide dismutase (SOD)

SOD was analyzed using alkaline pyragallol method described by Marklund and Marklund, (1974). Briefly, 950 µl of 100 mM Tris-Cl (pH 8.0) was combined with 50 µl of 6 mM pyrogallol, prepared in 0.1M HCl. The initial OD was set to 0.03. An appropriate aliquot of the crude enzyme was added and inhibition of the auto-oxidation monitred. One unit of SOD was defined as the amount of enzyme that causes 50 % inhibition of the pyrogallol auto-oxidation rate at 420 nm. Protein concentration was determined by Bradford method (Bradford, 1976) for calculating specific activity.

b) Catalase

Catalase activity was measured spectrophotometrically by monitoring the decrease in absorbance at 240 nm due to decomposition of hydrogen peroxide in 100 mM potassium phosphate buffer, pH 7.0 at 25 °C. Briefly, 900 µl of 100mM potassium

phosphate buffer, pH 7.0 was combined with 50 µl of 0.7 % H_2O_2 such that the initial OD is set at 0.4. An appropriate aliquot of crude extract was added and decrease in absorbance is recorded at an interval of 10s. One unit of the catalase was defined as the disappearance of 1 µmol of hydrogen peroxide (Σ= 0.041 mM^{-1} cm^{-1}) (Yun and Lee, 2000). Protein concentration was determined by Bradford method (Bradford, 1976) for calculating specific activity.

3.2.8 Activity staining of ROS reactive combative enzymes

a) Superoxide dismutase (SOD)

SOD activity staining done by method described by Yun and Lee, (2001). Briefly, proteins were resolved by 10 % non-denaturing polyacrylamide gel electrophoresis (PAGE) in Tris-glycine buffer. The gels were soaked in 490 µM NBT for 20 min, then in a solution containing equal volumes of 28 mM TEMED, 28 µM riboflavin and 36 mM potassium phosphate buffer (pH 7.8) for 15 min. The gels were subsequently illuminated with a fluorescent lamp for 5-15 min to visualize white bands of SOD activity on the blue background.

b) Catalase

Proteins in cell-free extract were resolved by 10 % non-denaturing polyacrylamide gel electrophoresis (PAGE) in Tris-glycine buffer. Gels were incubated initially with 5 mM hydrogen peroxide followed by a freshly prepared mixture of 2 % ferric chloride and 2 % potassium ferric cyanide. Catalase bands were visible as yellow bands against a green background (Yun and Lee, 2000).

3.2.9 Construction of pRADZ3-precA

*rec*A promoter (p*rec*A) from DR1 was PCR amplified using the forward primer containing *Bgl*II site P*rec*AF: 5′-CATG*AGATCT*CCGGTTGCCGTAAAGCT-3′ (*Bgl*II underlined) and the reverse primer containing *Spe*I site P*rec*AR: 5′CTTC*ACTAGT*CCCCGTTCGCCCAGTTC-3′ (*Spe*I site underlined). PCR was carried out in 30 µl reaction mixture consisting of 1ng of DR1 genomic DNA, 30 pmol. of each of the primers, 1 µl of mixture of dNTPs containing 2.5 mM each, 1.5 U of *Taq.* DNA polymerase combined with appropriate amount of 10X *Taq.* DNA polymerase buffer. Amplification was carried out in AB Biosystems thermal cycler (CA., USA). The PCR reaction was carried with an initial denaturation at 94 °C for 5 min.followed by 30 cycles each consisting of denaturation at 94 °C for 30 s.,

annealing at 50 °C for 30 s, elongation at 72 °C for 45 s, and a final extension at 72 °C for 10 min. The amplified products were analysed on 1 % agarose gel.

The amplified promoter was cloned in pTZ57R/T according to the manufacture's instruction (MBI Fermentas, Germany) and transformed in *E.coli* DH5α by $CaCl_2$ method described by Sambrook and Russell, (2001). For expression in DR1 the *rec A* promoter was sub cloned in the *Bgl*II and *Spe* I site of pRADZ3.

3.2.10 β-galactosidase assay

For quantitative analysis of *lac*Z expression, cells were permeabilized with Triton X-100 as follows. Samples (1 ml) of cultures at an OD_{600} 0.3 (or an equivalent volume) were centrifuged and the cell pellets were resuspended in 60 µl of lysis buffer (10 mM Tris-HCl pH 8, 1 mM EDTA, 100 mM NaCl, 1.5 % SDS (w/v), 2.5 % Triton X-100) (v/v), (Bonacossa de Almeida et al., 2002). The suspension was incubated for 10 min at 0 °C before β-galactosidase was assayed as described by Miller, (1982). The activity of *lac*Z was determined using a molar absorption coefficient of 0.0045 $nmol^{-1}$ ml^{-1} cm^{-1} at 420 nm. The protein concentration was determined by Bradford method (Bradford, 1976) for estimating specific activity.

$$\beta\text{-galactosidase specific activity} = \frac{OD420 \times 1.7}{0.0645 \times t \times v}$$

where 1.7 ml is the total volume of the assay mixture; t is time (min); v= volume of cells taken for the assay i.e. 1ml.

3.2.11 Quantification of total carbonylated protein

The total carbonyl content in cellular proteins was determined spectrophotometrically as described by Semchyshyn et al., (2005). Crude extracts were prepared fromDR1 cells treated or untreated with varied concentrations of Cd^{2+} for 12 h. Extracts were treated with streptomycin sulphate (2 %) and incubated on ice for 15 min. Precipitated nucleic acids were discarded by centrifugation at 14,000 g for 5 min. After adding four volumes of 10 mM dinitrophenylhydrazine (DNPH) prepared in 2 M HCl to 100 µl of the nucleic acid-free supernatant, the mixture was incubated for 1 h at room temperature with vortexing every 10–15 min. Proteins were precipitated by adding 500 µl of 20 % trichloroacetic acid (TCA) and then sedimented by centrifugation at 14,000 g for 5 min. The pellet was washed at least three times with an ethanol : ethylacetate mixture (1:1) to remove any unreacted DNPH and

dissolved at 37 °C with 450 µl 6 M guanidine HCl. Carbonyl content was determined spectrophotometrically at 370 nm using a molar absorption coefficient of 22,000 M^{-1} cm^{-1}(Semchyshyn et al., 2005).

3.2.12 Immunodetection of carbonylated proteins

DNPH derivatized protein extracts prepared as described in Section 3.2.11 were analyzed by 12 % SDS-PAGE, using 25 µg of derivitized protein per lane. Gels were either stained with Coomassie brilliant blue or transferred to nitrocellulose membrane filters using a tank blot system (Bangalore genei Pvt. Ltd., India). Electroblotting was done in a buffer system consisting of 25 mM Tris, 192 mM glycine, and 10 % methanol at 50 V for 3 h. (Harper and Speicher, 2001). The transferred proteins were detected using 0.5 % Ponceau S (prepared in 5 % acetic acid). To detect the carbonylated proteins anti-dinitrophenyl (anti-DNP) antibodies (Sigma, St. Louis, USA) were used. The membrane was blocked for 1 h at RT using 2 % skimmed milk prepared in phosphate buffered saline, (PBS) (137 mM NaCl, 2.7 mM NaCl, 100 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4). Immunodetection was done using mouse monoclonal anti- DNP antibodies at 1:5000 dilution for 1 h at RT. Unbound antibody was washed using 0.005 % Tween-20 in PBS by gently rocking the membrane for 1 min. This step was repeated thrice. Goat anti-mouse IgG- peroxidase conjugate (Bangalore Genei Pvt. Ltd., Bangalore, India) was used at 1:5000 dilution as secondary antibody. The excess of antibody was washed using 0.005 % Tween-20 in PBS. The carbonylated proteins were then detected by treating the membrane with 5 mg 3′,5′, di aminobenzidine (DAB) dissolved in 10 ml PBS containing 2 mg $NiCl_2$ and 10 µl 30 % H_2O_2 .The carbonylated protein appeared as brown bands (Gallagher, 2001).

3.2.13 Determination of thiobarbituric acid-reactive substances (TBARs)

TBARs in cell extracts were determined as described by Semchyshyn et al., (2005). The cultures of DR1 were grown in presence of varying concentration of Cd^{2+} in a volume of 600 ml for 12 h. The cells were collected by centrifugation at 9560 x g for 10 min, washed with 0.1 volumes of PBS, concentrated by centrifugation and resuspended in 0.05 volume of PBS. A 1ml aliquot of cell suspensions was treated with 1 ml of 20 % TCA (w/v) and centrifuged at 10,000 g for 5 min. Supernatants were mixed with 2.0 ml of a saturated solution containing thiobarbituric acid (0.67 %,

w/v) prepared in 0.1 M HCl. Samples were then heated for 60 min in a water bath kept at 100 °C. Aliquots of 1.5 ml were then removed, chilled, mixed with 1.5 ml of butanol and centrifuged at 4,000 g for 10 min. The organic fraction was recovered and the OD_{535} was measured spectrophotometrically. TBARs content was determined using a molar extinction coefficient of 156 mM^{-1} cm^{-1} (Semchyshyn et al., 2005).

3.2.14 Metal binding protein preparation by Immobilised metal affinity chromatography (IMAC)

A 5 ml syringe (6.5 cm x 1.5 cm) was packed with glasswool to which 1 ml of the Imino-diacetic acid (IDA- Agarose (Biorad Ltd, CA, USA) was pipetted using cut tips. The column was equilibrated with 5 column volumes of solution C (50 mM sodium acetate, 0.3 M NaCl, pH 4.0) and 5 column volumes of 0.3 M solution of either $MnCl_2$ or $CdCl_2$was applied to the column to charge the column of appropriate metal solution, while the control column was washed with equal volume of solution C. The columns were washed with solution C to remove any excess of metal solution. The column was then washed with 10 column volumes of deionised water and allowed to equilibrate with at least 5 column volumes of solution A (50mM sodium phosphate, 0.3 M NaCl, pH 8) (Bio-rad, CA, USA).

DR1cells grown in a volume of 600 ml at different conditions were harvested at 12,300 g for 10 min and frozen at -20 ^{o}C till used. The thawed cells were resuspended in 1:10 (w/v) in solution A and then disrupted using French press at 1000 psi. The suspension of disrupted cells was centrifuged at 12,300 g for 10 min. The supernatant was collected and was filtered using 0.45 μm filter. IDA-agarose column charged with appropriate metal was used for purification of the protein. Crude cell lysate (900 μg protein) was loaded on IDA-agarose column, washed with solution A till OD_{280nm} 0.001 was obtained. Elution of protein was done using 5 bed volumes of solution B (solution A + 500 mM Immidazole) at a flow rate of 1ml/min. Eluted protein fractions were resolved on 12 % SDS-PAGE and detected by silver staining (Sambrook and Russell, 2001). The gels were analysed using the online software, BIONUMERICS (Applied Maths, Belgium). The fractions containing maximum protein were pooled together and dialyzed for 24 h against 10 mM Tris-Cl pH 8.0. The samples were lyophilized and resuspended in minimum volume of distilled water.

The columns were recharged by washing with 10 bed volumes of eluting solution (solution A + 0.5 M EDTA) to strip the column from any adhering metal. Five bed

volume of 1 M NaOH was used for rinsing the column to remove precipitated, hydrophobic and lipoproteins. The column was rinsed with distilled water till pH 7.0 was obtained. Washing was done with 10 bed volume of solution A.

3.2.15 Two- Dimensional gel electrophoresis (2-DE)

DR1cells grown in a volume of 600 ml at different conditions were harvested at 12,300 g for 10 min and frozen at -20 ^{o}C till used. The thawed cells were resuspended in 1:10 (w/v) in Tris-buffered sucrose (10 mM Tris-Cl pH 7.0; 250 mM sucrose). Washed cells were disrupted using French press at 1000 psi. The cell lysate was briefly sonicated for 3 min (9.9 s on and 9.9 s off) to shred off DNA. The lysate was clarified by centrifugation at 12,300 g for 15 min at 4 °C and filtered through 0.45 µm membrane filter. The homogenates were treated with 10 µg/ml each of DNAase and RNAase, and incubated at 4 °C for 12h. The samples were then dialysed against 1 mM Tris-Cl pH 7.0 for 3 h followed by concentration by lyophilisation. The proteins were resuspended in 500 µL of distilled water. Protein concentration was estimated by Bradfords method (Bradford, 1976).

2-DE was performed according to the manufacturer's instruction (Bio-rad, CA, USA). Briefly, each protein sample, 25 µg, in the lysis buffer was diluted to 125 µl with rehydration solution (9 M Urea, 2% CHAPS, 30 mM DTT, 0.5% ampholytes pH 3-10, 0.002% bromophenol blue). IEF dry strip gels (pH 4–7), 7 cm, (Bio-rad, CA, USA) were rehydrated with 125 µl of mixture solution in 7 cm strip holders and electrofocused with Protean (IEF) Isoelectric Focusing System (Bio-rad, CA, USA). The focusing protocol was performed as follows: 50 µA per strip at 20 °C; 250 V for 20 min; 4000 V for 2 h; and 15000 V/hr. After isoelectric focusing, strips were equilibrated (10 min) with gentle shaking in SDS equilibration buffer I (0.375 M Tris-Cl buffer, pH 8.8, 6 M urea, 20 % glycerol, 2 % SDS, 2 % DTT w/v), SDS equilibration buffer II (0.375M Tris- Cl buffer, pH 8.8, 6 M urea, 20 % glycerol, 2 % SDS, 2 % DTT, 2.5 % iodoacetamide w/v), then loaded onto SDS PAGE (12 %). The second dimension SDS electrophoresis was performed using Mini-PROTEAN SYSTEM. (Bio-rad, CA, USA). The protein spots were detected by silver staining (Sambrook and Russell, 2001) and the gels were compared using online 2D comparing software, Melanie 7.0 (Swiss Institute of Bioinformatics, Geneva, Switzerland).

3.3. Results and Discussion

3.3.1 Metal tolerance of radiation resistant bacteria

For the potential use of radiation resistant bacteria for the remediation of nuclear waste, the tolerance of the bacteria towards heavy metals, which are often accompanying the radionuclides in the dump areas, is important. The most common heavy metal contaminants at the radioactive waste sites are Cd^{2+}, Hg^{2+}, Cr^{6+} at varying concentration (NABIR Primer 3; *http://www.lbl.gov/NABIR*). Hence the tolerance of deinococci to the common metal contaminants was determined.

3.3.1a Tolerance to Hg^{2+}

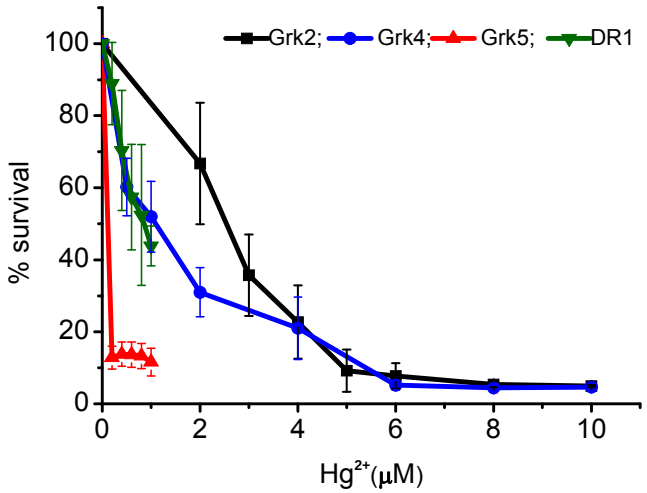
The effect of Hg^{2+} was examined on the radiation resistant bacteria wherein the cultures showed varying level of sensitivity to Hg^{2+} (Fig. 3.1). DR1 exhibited extreme sensitivity to Hg^{2+} while the isolate Grk2 showed a maximum D_{50} of 2.08μM, 2.3 fold higher than DR1. The highest reported concentration of Hg^{2+} at the radioactive waste sites is 10 μM (Lange et al., 1998). DR1 has been engineered to express the *mer*A gene of *E. coli* BL308 and it results in tolerance upto 30 μM Hg^{2+} (Brim et al., 2000). *E. coli* shows a minimum inhibitory concentration (MIC) of 10μM in minimal media (Nies et al., 1999) while *Ralstonia metallidurans* CH34 exhibits a MIC of 50 μM in minimal media (Dressler et al., 1991). Therefore DR1 is extremely sensitive to Hg^{2+} while Grk2 can be considered moderately resistant to Hg^{2+}.

3.3.1b Tolerance to Cr^{6+}

Chromium mainly occurs as Cr^{6+} in the divalent oxyanion chromate and as Cr^{3+}, the trivalent cation. Cr^{6+} which is more toxic than Cr^{3+} (Nies et al., 1999) is taken up by microbial cells as CrO_4^{2-} via sulfate (SO_4^{2-}) transport mechanisms (Silver and Phung, 2005). The Cr^{6+}tolerance of the radiation resistant bacteria revealed that DR1 showed a D_{50} upto 252 μM while the isolates showed considerably lower tolerance to Cr^{6+} (Fig. 3.2). The MIC of *E.coli* for Cr^{6+} has been reported to be 200 μM in minimal media (Nies et al., 1999). DR1 has been demonstrated to reduce Cr^{6+} with humic acid as final electron acceptor (Fredickson et al., 2000). Also complete mineralization of toluene by the bioengineered strain of DR1 with concomitant reduction of Cr^{6+} has been reported (Brim et al., 2006).

3.3.1c Tolerance to Cd^{2+}

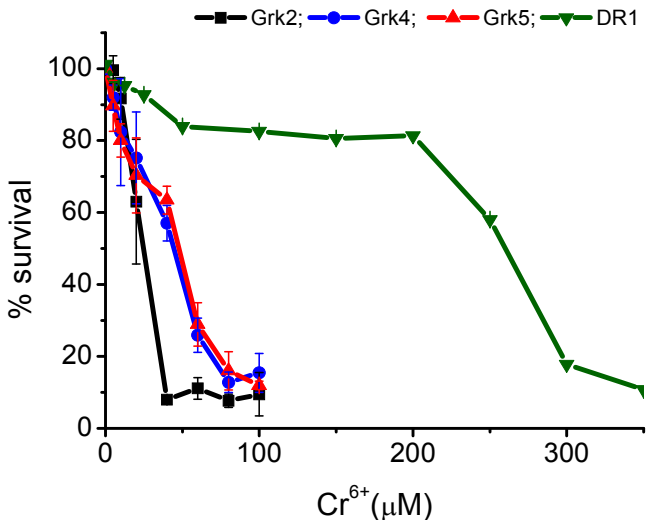
Cadmium is also thiol-binding metal like Hg^{2+}. The toxic effects of Cd^{2+} is summed up under the general headings "thiol-binding and protein denaturation, interaction with calcium metabolism and membrane damage and interaction with zinc metabolism, or loss of a protective function" (Neis et al., 1999; Cuypers et al., 2010). Only in rare cases has an important single mechanism been found (Stohs and Bagchi, 1995; Cuypers et al, 2010). The deinococcal isolates and the type strain DR1 exhibited varying levels of tolerance, Grk2 being extremely sensitive to Cd^{2+} (Fig. 3.3). DR1 being moderately tolerant while Grk4 and Grk5 showed comparable tolerance. However, when compared to *E.coli* that shows a MIC of 500 µM; all the deinococcal strains can be considered sensitive to Cd^{2+} (Nies et al., 1999).



	D. radiodurans R1	Grk2	Grk4	Grk5
D_{50} (µM)	0.9 ±0.005	2.08± 0.59	1.23±0.34	0.078±0.26

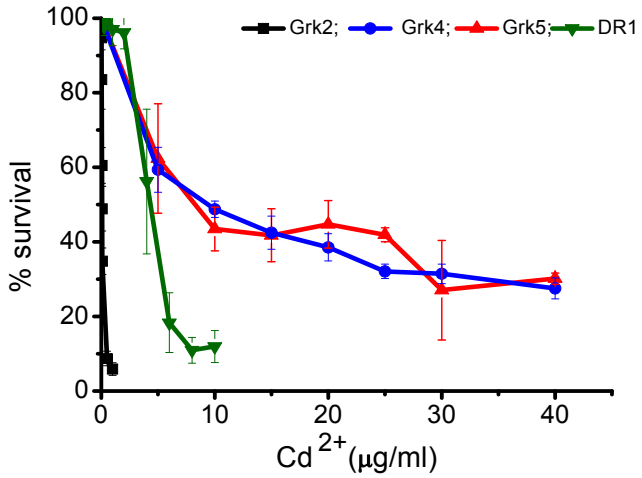
Fig. 3.1: D_{50} determination of deinococcal strains to Hg^{2+}. D_{50} was determined as the concentration that results in the reduction in survival by 50 % of the control. Each point represents an average of three independent experiments. Table lists the D_{50} of all the isolates.

Ruggerio et al., (2005) presented a comprehensive analysis of the effect of heavy metals and actinide on DR1 and *P. putida,* wherein they reported inhibitory concentration of 1.8 µM Cd^{2+} for DR1 while *P. putida* under similar condition could tolerate upto 500 µM. However both DR1 and *P. putida* showed comparable resistance to Cr^{6+} (Table 3.2).



	D. radiodurans R1	Grk2	Grk4	Grk5
D_{50} (μM)	252.14±23	24.54±4.64	45.89± 3.59	48.36 ±2.19

Fig. 3.2: D_{50} determination of deinococcal strains to Cr^{6+}. D_{50} was determined as the concentration that results in the reduction in survival by 50 % of the control. Each point represents an average of three independent experiments. Table lists the D_{50} of all the isolates.



	D.radiodurans R1	Grk2	Grk4	Grk5
D_{50} (μg/ml)	3.91±0.36 (21)	0.076±0.004 (0.41)	10.53± 0.37 (57)	9.48 ± 0.58 (51.7)

Fig. 3.3 D_{50} determination of deinococcal strains to Cd^{2+}. D_{50} was determined as the concentration that results in the reduction in survival by 50 % of the control. Each point represents an average of three independent experiments. Table below lists the D_{50} of all the isolates. Values indicated in parentheses represent the corresponding μM concentrations.

Table 3.2 Comparative heavy metal resistance of radiation resistant bacteria, *E.coli* and *P.putida*

	Hg^{2+} (µM)	Cr^{6+} (µM)	Cd^{2+} (µM)	Reference
D. radiodurans R1	0.4	225	17 (3.28)	This study
Grk2	1.88	17.83	0.049 (0.009)	This study
Grk4	0.38	24.1	21 (3.9)	This study
Grk5	0.05	24.7	23 (4.34)	This study
E.coli[a]	10	200	500	Nies et al., (1999)
P.putida [b]	ND	100	530	Ruggerio et al., (2005)

† D_{70} values of deinococcal isolates for each of the metals are reported. The values in parentheses indicate the D_{70} for Cd^{2+} in µg/ml

[a]Refers to MIC determined in minimal media.

[b]Minimum concentration of metal that causes >70% growth inhibition.

3.3.2 Effect of growth phases on Cd^{2+} toxicity

In nature, bacteria can survive for long periods in non-growing stationary states. Changes in morphology and physiology that occur in the stationary-phase bacteria and concomitantly a state of increased resistance against various stresses are established (Ishihama, 1999; Nystrom, 2004).

During the course of our work we observed the ability of DR1 to tolerate Cd^{2+} depended on the growth phase. Contrary to general observations, the exponentially growing cultures were able to tolerate higher concentration of Cd^{2+} as opposed to stationary phase culture when used as inocula in Cd^{2+} containing media (Fig. 3.4). In DR1, the log phase culture when inoculated in Cd^{2+} supplemented media, the culture did not show any growth inhibition (Fig. 3.4A), while the stationary phase inoculum was completely inhibited under the same Cd^{2+} concentration (Fig. 3.4B). The log phase culture grown in presence of Mn^{2+} exhibited Mn^{2+} induced cell division (MnCD), characteristic of DR1 (Chou and Tan, 1990). Growth in presence of Mn^{2+} and Cd^{2+} showed the dominant growth pattern of Mn^{2+} for log phase culture exhibiting a slight delay in MnCD effect, while Cd^{2+} growth pattern dominated for stationary phase culture. The Cd^{2+} D_{50} of exponential phase culture was determined to be 3.3 µg/ml whereas that of the stationary phase culture was 1.3 µg/ml (Fig. 3.5). The D_{50} value of Cd^{2+} in presence of Mn^{2+} increased and was comparable to log phase cells (Fig. 3.5). The exponential phase culture of DR1 did not show any stasis

however, the stationary phase culture of DR1 underwent stasis of 48 h before resuming growth (Fig. 3.6)

Microscopic examination of the DR1cells 3 h post stasis, recovering from Cd^{2+} stress showed departure from the usual diplococci or tetracocci morphology of DR1, and exhibited lysed morphology. However the control cells showed the characteristic tetrad throughout the growth phase. After 6 h post recovery the cells exhibited diplococcic or teteracocci morphology but some unstained patches were observed in all the cells examined (Fig. 3.7), indicating cell envelop alterations. Ferianc et al., (1998) reported the lag phase cultures of *E.coli* could tolerate up to 3 µM Cd^{2+} with complete growth inhibition at 10 µM Cd^{2+} in minimal media as opposed to exponential phase culture that could tolerate up to 273 µM Cd^{2+}. Similarly log phase cultures of DR1 are 2.5 fold more resistant to Cd^{2+} than the stationary phase cultures.

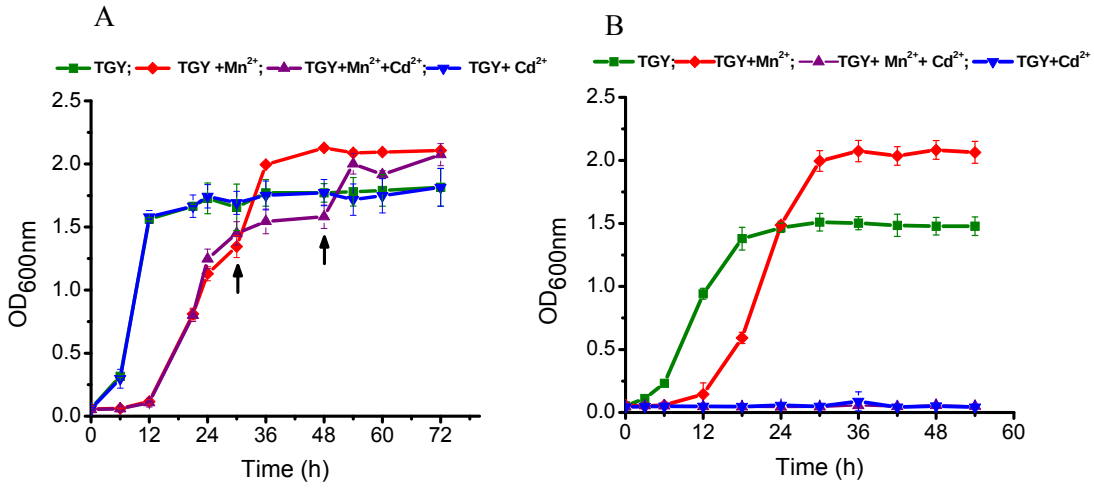
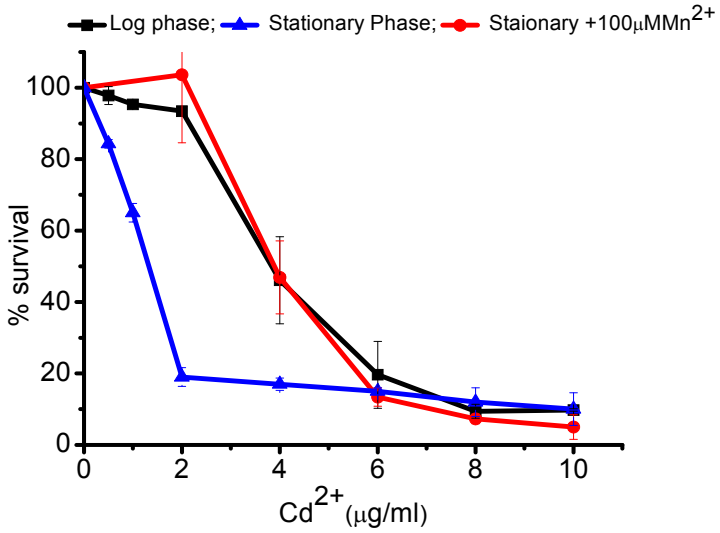


Fig. 3.4:Effect of growth phase on Cd2+ tolerance in DR1.The medium was supplemented with 1.2 µg/ml Cd^{2+}; 100 µM Mn^{2+} or both while no metal amendment served as control. A) Exponential phase; B) Stationary phase. Arrows indicate the MnCD effect.

DR1 has been shown to accumulate Mn^{2+} intracellularly which protects the important biomolecules from lethal effects of ionising radiation (Ghosal et al., 2005). In several gram positive bacteria Cd^{2+} toxicity has been affected through Mn^{2+} transporters and is known to be potent inhibitors for Mn^{2+} transporters (Hao et al., 1999; Makui et al., 2000; Keheres et al., 2000; Horsburgh et al., 2002). DR1 has two forms of Mn^{2+} transporters, Nramp Mn^{2+} transporters similar to the *mnt*H transporters identified in *E.oli* and *S. typhimurium* and ABC type transporters (Daly et al., 2004).



	DR1 log phase	DR1 Stationary phase	DR1 Stationary phase + 100 μM Mn^{2+}
D_{50} (μg/ml)	3.9± 0.36(21)	1.21±0.02 (6.6)	3.8±0.48(20)

Fig. 3.5: Effect of exogenous Mn^{2+} addition to Cd^{2+} toxicity to stationary phase cultures in DR1. Table below indicate the D_{50} value of DR1 under different growth conditions. Values in parentheses indicate μM concentration.

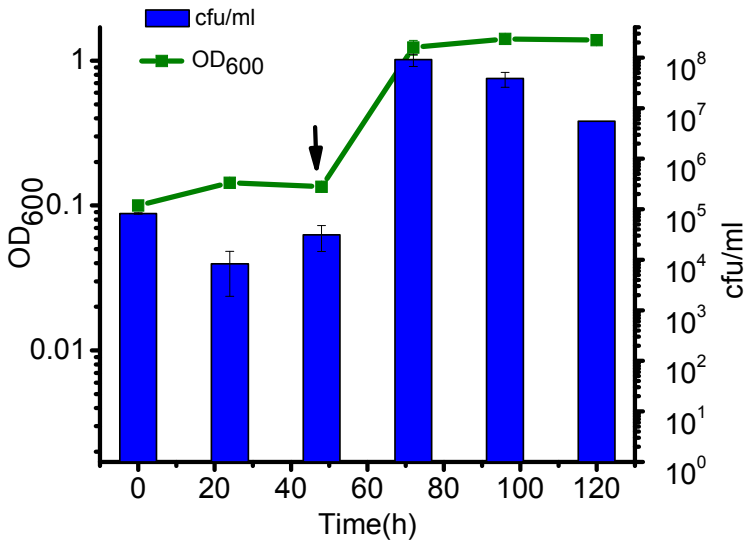


Fig. 3.6: Cd^{2+} induced stasis of the stationary phase culture of DR1. Arrow indicates the point of growth resumption.

The enhanced tolerance of Cd^{2+} in stationary phase culture of DR1 in presence of Mn^{2+} (Fig. 3.5) indicates that Cd^{2+} proves to be a competitive substrate for the Mn^{2+} transporters in DR1. An increased tolerance towards Cd^{2+} also affirms that Mn^{2+} ions are able to antagonize the transport of Cd^{2+}.

As opposed to the exponentially growing cultures of *E.coli* that undergo stasis when challenged with Cd^{2+} stress (Ferianc et al., 1998), stationary phase cultures of DR1 exhibited stasis (Fig. 3.6). In congruence with *E. coli* cultures, that requires synthesis of new proteins for recovery after exposure to Cd^{2+} (Mitra, 1984). DR1 cells treated with chloramphenicol failed to recover from Cd^{2+} induced stasis (data not shown). This affirms the fact that DR1 requires new protein synthesis to recover from stasis induced by Cd^{2+}. Additionally transcriptome analysis of *E.coli* cells exposed to Cd^{2+} revealed down regulation of several ribosomal proteins. All genes for protein translation machinery are down-regulated, after which some resumed expression in the late phase, while genes for stress proteins were mostly up-regulated consistent with the decline in the overall rate of protein synthesis (Wang et al., 2005).

Heavy metals such as Cu^{2+}, Co^{2+}, Hg^{2+} and Cd^{2+} are known to induce morphological changes leading to change in cell elongation (Chakarvarty and Banerjee, 2008; Antony et al., 2011; White and Gadd, 1998; El-Rab et al., 2006). However at higher concentrations, Co^{2+} and Hg^{2+} cause lysis of the cells (Vaituzis et al., 1975, Antony et al., 2011). Fig. 3.7 demonstrates that Cd^{2+} also affects morphological changes in recovering cells of DR1. Massalki et al., (1981) demonstrated that the cells of the green alga *Ankistrodesmus braunii* when exposed to Cd^{2+} showed the presence of multinucleate gaint cells formed due to continuous mitotic division without subsequent cytokinesis. The nuclei of such giant cells exhibited the presence of deep indentations that appeared hole like structures. Similar structures were also observed in DR1 cells that did not exhibit normal morphology (Fig. 3.7B). Recently, Joe et al., (2011) studied the transcriptional profile of DR1 exposed to toxic levels of Cd^{2+}. Among other genes, there was a down regulation of the expression of genes involved in biosynthesis of murein sacculus, surface polysaccharides, lipopolysaccharides and surface structures.

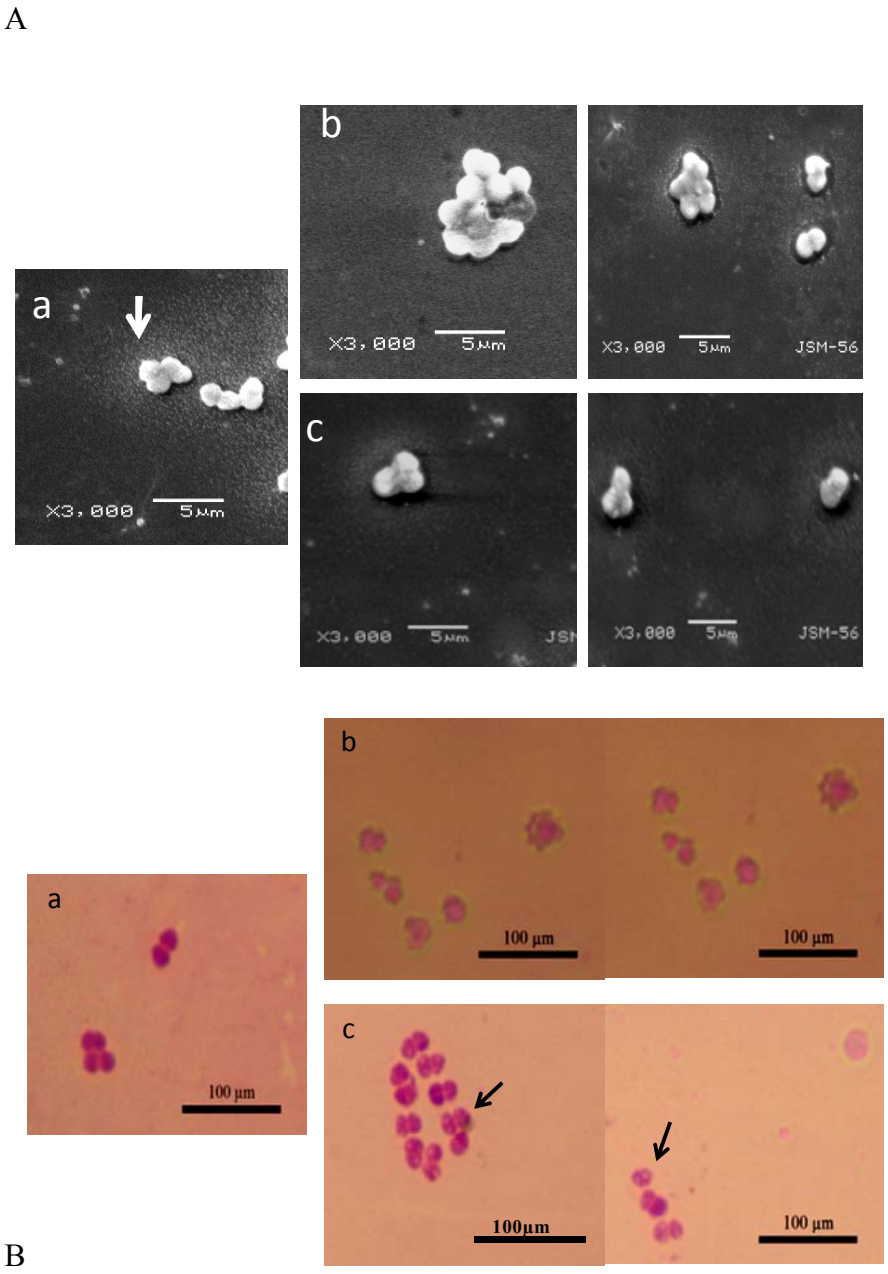


Fig. 3.7: Micrographs of the Cd^{2+} treated DR1after assuming growth A) Scanning electron microscopy; B) Light microscopy of gram stained DR1; a) Control, the arrow shows normal tetrad of the DR1; b) 3 h post stasis; c) 6 h post stasis. The arrow represents the unstained patches on the cells.

3.3.3a Cd^{2+} and reactive oxygen species (ROS) generation

Chemically reduced and acetylated 2′, 7′-dichloro hydrofluorescein diacetate (H_2DCFDA) is a non-fluorescent dye that is freely permeable to the cells. Once inside the cells, it is hydrolysed to 2′, 7′ dichlorofluoroscein (DCF) and trapped

intracellularly, DCF is then able to interact with peroxides resulting in fluorescent, 2′, 7′-dichlorohydrofluorescein.The fluorescent probe H_2DCFDA was used to monitor formation of intracellular ROS in Cd^{2+} treated cells of DR1 (Fig. 3.8). Cd^{2+} induces oxidative stress at sub-lethal concentration; however there is a decrease in intra-cellular ROS at D_{50} concentration of Cd^{2+} that can be attributed to increased lethality by increasing Cd^{2+} concentration (Fig. 3.9 (A)). A similar effect was seen with H_2O_2 wherein sub-lethal concentration elicits intracellular ROS that declines at higher concentration of H_2O_2 known to be lethal in DR1 Fig. 3.9 (B) (Wang and Schellorn, 1995). The ROS produced by 10 μM Cd^{2+} was similar to that produce by 60 mM H_2O_2 (Fig. 3.10).

Metal catalyzed oxidation and generation of oxidative stress *in vivo* is a commonly observed phenomenon for redox-active metals such as Fe^{2+} and Cu^{2+}. Although Cd^{2+} is considered to be a redox inactive metal, yet it has been shown to cause protein carbonylation a common index for the ROS induced damage (Stohs and Bagchi, 1995; Cuypers et al., 2010). The Cd^{2+} induced ROS demonstrated here could be a direct effect of Cd^{2+} or indirect by replacement of Fe from Fe-S cluster that can promote further Fenton type chemistry producing hydroxyl radical (Cuypers et al., 2010).

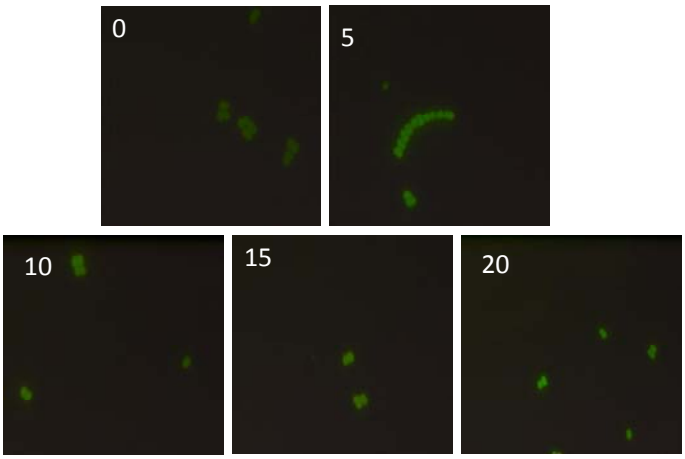


Fig. 3.8: Fluorescence micrograph of Cd^{2+} exposed DR1 cells stained with H_2DCFDA.
Numbers indicate Cd^{2+} concentration in μM.

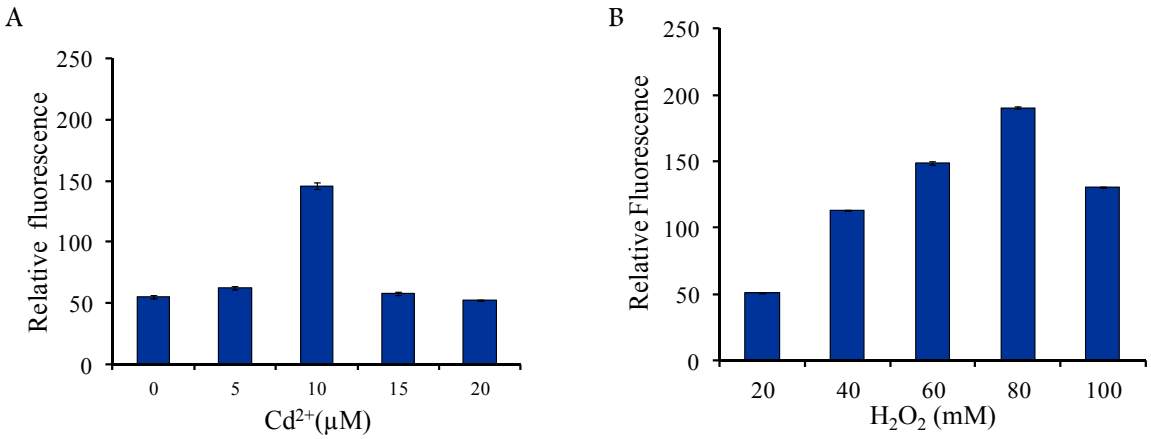


Fig. 3.9: Effect of Cd^{2+} and H_2O_2 on ROS generation in DR1. Relative fluorescence obtained by H_2DCFDA treated cells A) after Cd^{2+}treatment, B) after H_2O_2 treatment. Fluorescence obtained was normalised using the protein content after each treatment to obtain relative fluorescence.

3.3.3b Induction of protein carbonylation by Cd^{2+}

ROS has multiple targets in the cell with all biomolecules being affected by it (Imlay, 2003; Avery, 2011). Protein carbonyl derivatives are formed by direct metal catalyzed oxidative (MCO) attack on the amino-acid side chains of proline, arginine, lysine, and threonine (Nystrom, 2005). The carbonylation of the protein renders the proteins inactive and more susceptible to degradation. In DR1, the protein carbonylation increased with increasing Cd^{2+} concentration which is congruence with the ROS that is produced (Fig. 3.10). However, a decrease in ROS activity at higher concentration but sustained levels of carbonylated protein was observed indicating that at higher concentrations Cd^{2+} may impair the degradative capacity of DR1. Immunoblot assay also reveals protein oxidation increases with increasing Cd^{2+} concentration (Fig. 3.11). Although the ROS declines beyond 10 µ M Cd^{2+} there is sustained presence of carbonylated proteins. A diminished degradative capacity of the cell may contribute to the persistence carbonylated protein in the cell (Nystrom, 2005).

3.3.3c Effect of Cd^{2+} on lipid peroxidation

Peroxidation of membrane lipids is a complex process involving unsaturated fatty acids and in particular, polyunsaturated fatty acids containing more than one methylene groups which are highly reactive to oxidizing agents. The oxidation can form peroxyl radicals that can set off a free radical chain reaction to other methylene groups and generate peroxidation by-products which could promote the loss of

integrity in the plasma membrane and, eventually, lead to cell death (Perez et al., 2007).

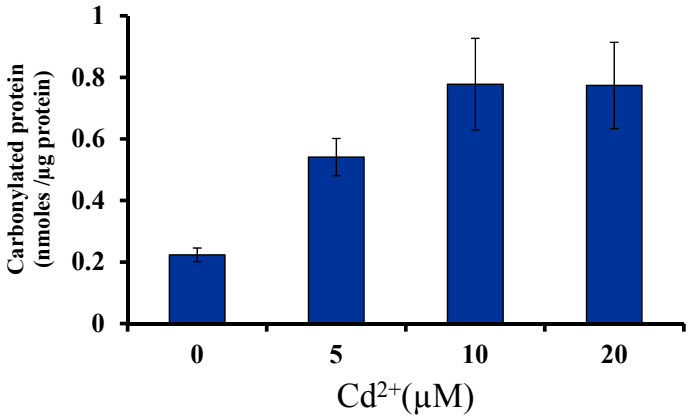


Fig. 3.10: Effect of Cd^{2+} on carbonylated proteins in DR1

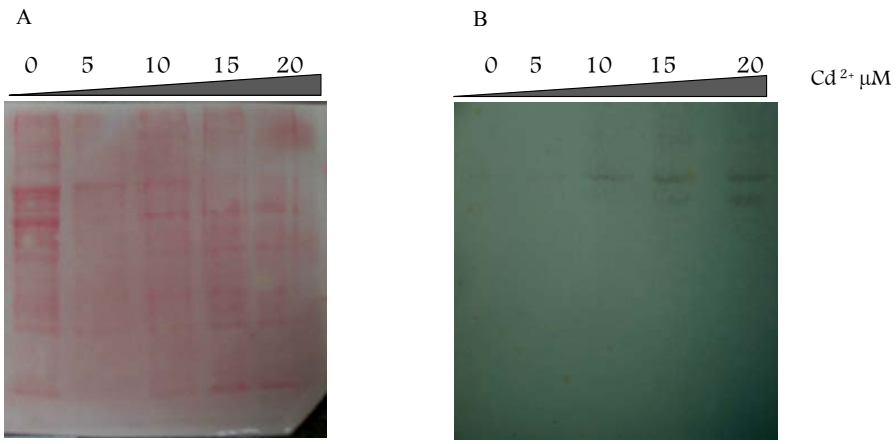


Fig. 3.11: Detection of carbonylated proteins in DR1 exposed to Cd2+. *A) Total cellular protein detection using Ponceau S; B) Immunodetection of carbonylated proteins by anti-DNP antibody.*

In DR1, Cd^{2+} induced the formation of lipid hydroperoxides, detected as TBARs, at all concentrations tested with a maximum obtained at 10µM (Fig. 3.12).

The TBARs detect primarily products arising from the decomposition of lipid hydroperoxides and is a common method to assess the damage to the lipids caused by free radicals (Howlett and Avery, 1997). Metals that promote Fenton type chemistry such as Cu^{2+} and H_2O_2 cause lipid peroxidation (Perez et al., 2007; Semchyshyn et al., 2005; Howlett and Avery; 1997). Howlett and Avery, (1997) demonstrated the lipid composition influenced the degree of lipid peroxidation with unsaturated fatty acids being more susceptible for lipid peroxidation. Fig. 3.12 demonstrates lipid peroxidation in DR1 which is in correlation with ROS production. The lipid

peroxidation due to Cd^{2+} was not dose dependent, a similar effect was reported in *Allium cepa* and *Vicia fabia* followed by Cd^{2+} exposure (Unyayar et al., 2006).

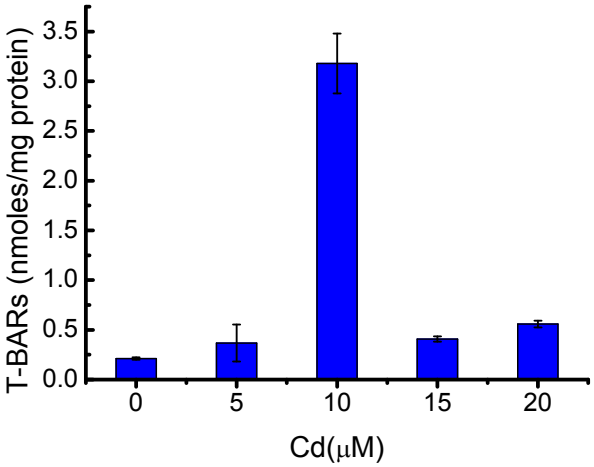


Fig. 3.12: Cd^{2+} induced lipid peroxidation in DR1

3.3.4 Effect of Mn^{2+} and Cd^{2+} on recovery from H_2O_2 stress in DR1

During starvation genes encoding proteins with specific roles in protecting the cell against external stresses, e.g., heat, oxidants, osmotic challenge, and exposure to toxic chemicals are expressed and these are the likely candidates required for starvation survival (Nystrom, 1999; Ishihama, 1997). Consequently, starved cells are highly resistant to a variety of secondary stresses, a phenomenon known as stasis-induced cross protection (Nystrom, 2004). It was hypothesised that inability of DR1 to tolerate Cd^{2+} in the stationary phase was due to the overwhelming oxidative stress that was produced by Cd^{2+}. Therefore we analysed the effect of Mn^{2+} and Cd^{2+} on cells recovering from oxidative stress imposed by treating the exponentially growing cultures to 20 mM H_2O_2 for different time intervals.

As seen in Fig. 3.13 (a) pre-treatment of H_2O_2 enhanced the survivability of the DR1 culture in presence of Cd^{2+} indicating the cross-resistance provided by H_2O_2 to Cd^{2+} stress. However as seen in Fig. 3.13(b), pre-treatment of Cd^{2+} was unable to provide any cross-resistance to H_2O_2 exposed cells while Mn^{2+} exhibited adaptability to either stress. To investigate the potential role of SOD in Cd^{2+} toxicity in DR1, *sod* A^- (Mn SOD) disruptant mutant of DR1 was subjected to Cd^{2+} stress. The mutants were found to be 5 times as sensitive to Cd^{2+} as compared to wild type, while Mn^{2+} amendment to *sod* A^- further aggravated Cd^{2+} toxicity (Fig. 3.14) which is in contrast

to that observed for wild type (Fig. 3.15). Addition of Mn^{2+} to DR1cells demonstrates high SOD as well as catalase activity indicating the ROS stress (Chou and Tan, 2000). Furthermore addition of Mn^{2+} induces the Embden-Merenhof pathway (EMP) as opposed to the Pentose Phosphate pathway (PP) leading to massive glucose oxidation that creates oxidative stress and exhaustion of the reducing power of the cell (Zhang et al., 2005). This might explain the higher catalase and superoxide dismutase (SOD) requirement after exposure to high levels of Mn^{2+} as well as the higher sensitivity of sod A^- mutant to Cd^{2+} in presence of Mn^{2+}.

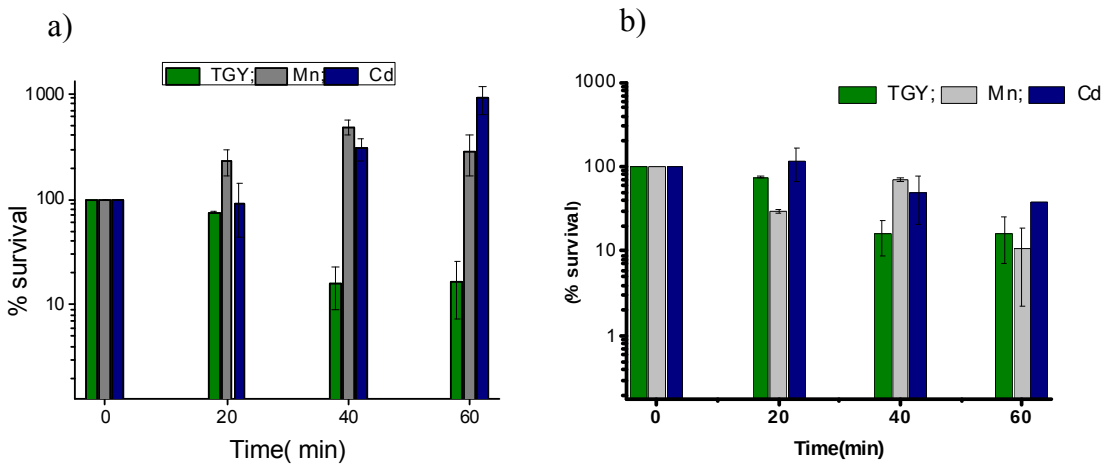


Fig. 3.13: Effect of Mn^{2+} and Cd^{2+} on recovery of DR1 from H_2O_2. a) cells are exposed to 20 mM H_2O_2 followed by recovery on TGY or TGY+100 µM Mn^{2+} or TGY+ 2.5 µM Cd^{2+}; b) Cultures are grown in TGY or TGY+100 µM Mn^{2+} or TGY+ 2.5 µM Cd^{2+}, exposed to 20 mM H_2O_2 followed by recovery on TGY plates.

3.3.5 Growth phase dependent production and influence of Cd^{2+} on anti-oxidative enzymes

The normal metabolism of aerobic cells also contributes to the ROS that is efficiently controlled by ROS combative enzymes produced by the cells (Imlay, 2003). The time course for the expression of catalase and SOD in DR1 under normal growth conditions was investigated. SOD activity was in good agreement to the values reported by Chou and Tan (1990) and the activity increased throughout the growth phase while showing a reduction of 75 % of the maximum activity obtained at 12 h, which was still 38 % greater than early log phase (Fig. 3.16 A).The catalase activity at 12 h, late exponential phase increased 10 fold as compared to the early exponential phase and decreased to the initial levels at early stationary phase (Fig. 3.16 B).

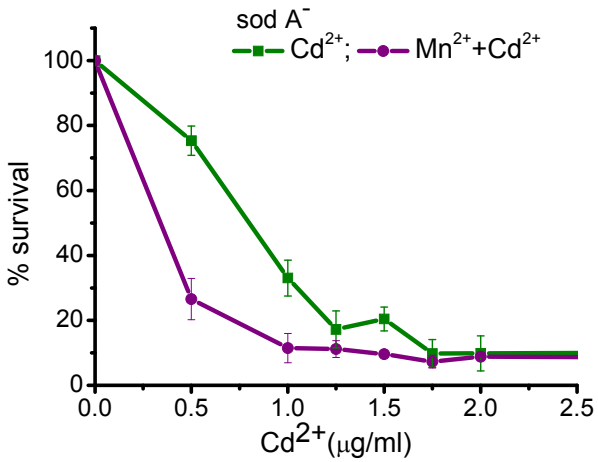


Fig. 3.14: Effect of Cd^{2+} on DR1 sod A^- mutant in presence and absence on 100μM Mn^{2+}.

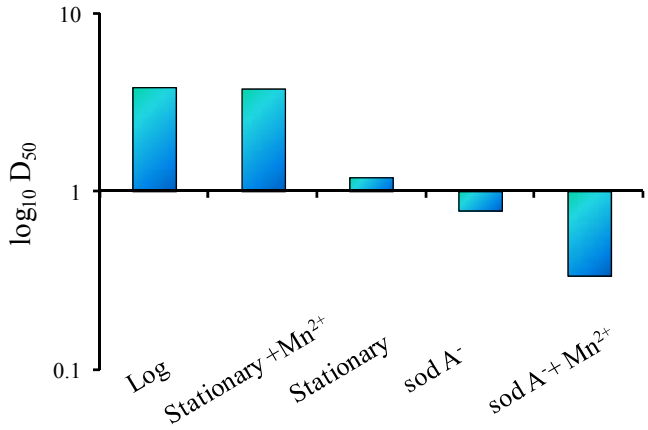


Fig. 3.15: A comparative analysis of Cd^{2+} D_{50} for the wild type DR1 grown under different growth phases and the DR1 sod A- mutant. The influence of Mn^{2+} on D_{50} for Cd^{2+} has been compared for both wild type and sod A^- mutant

The role of the two key anti-oxidative enzymes catalase and superoxide dismutase (SOD) toward Cd^{2+} toxicity was studied. Catalase was strongly inhibited even at the lowest concentration examined, 5 μM, showing a decrease of 80 % activity in comparison to control and was further corroborated by the activity staining (Fig. 3.17). This observation is supported by the fact the constitutive catalase in DR1 is Kat A, heme – containing enzyme that may be inactivated by the presence of the Cd^{2+} (Kobayashi et al., 2006).

No significant effect of Cd^{2+} on SOD activity was observed in the zymogram (Fig. 3.18 A). Nevertheless, activity measurements revealed a decline in the SOD activity at 5 µM Cd^{2+} and a complete inhibition at higher concentrations of Cd^{2+} treatment (Fig. 3.18 B). The *in vitro* effect of the Cd^{2+} on SOD enzyme activity from the Cd^{2+} untreated culture of DR1 showed that Cd^{2+} inhibited SOD activity by 10 % at 1 µM and 5 µM, while at 10 µM, 24 % reduction in the activity was observed.

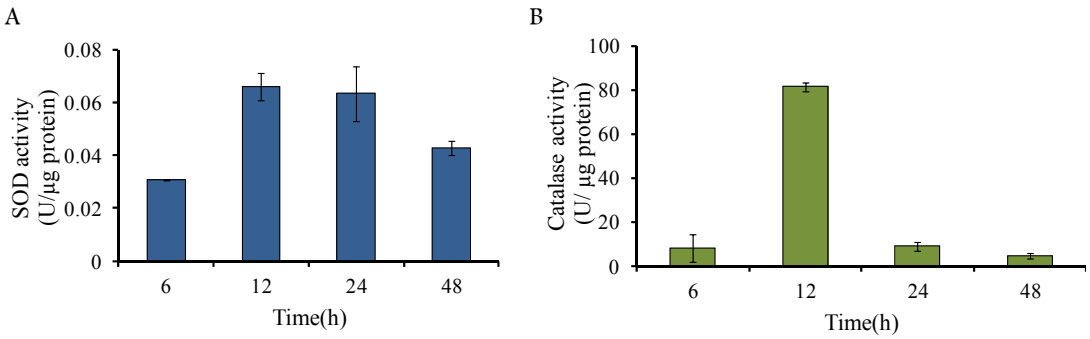


Fig. 3.16: Growth dependent expression of anti-oxidant enzymes in DR1 grown in TGY
A) SOD; B) Catalase

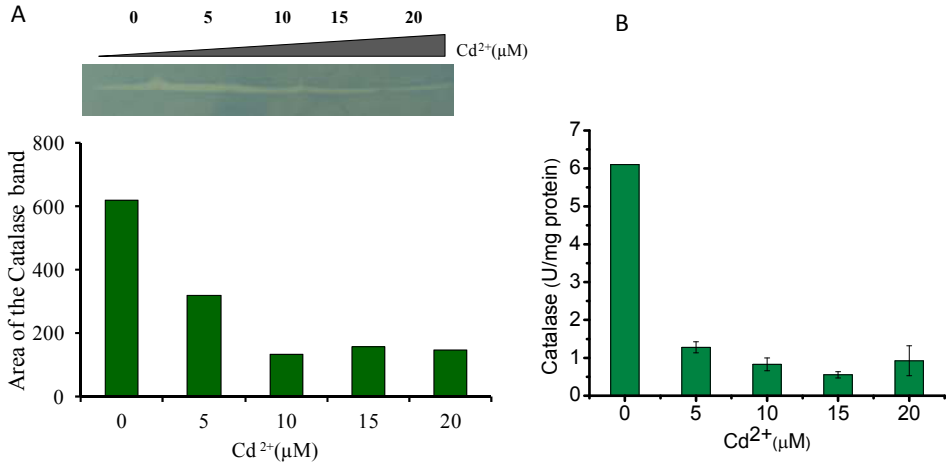


Fig. 3.17: Effect of Cd^{2+} on the catalase in DR1 A) Catalase zymogram and its densitometric scan; B) Activity of catalase in crude extract of DR1.

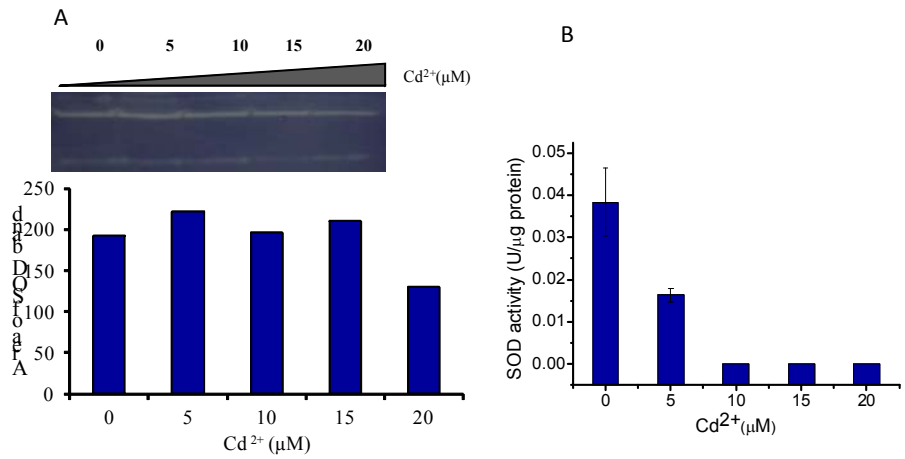


Fig. 3.18: Effect of Cd^{2+} on the SOD in DR1 A) SOD zymogram and its densitometric scan ; B) Activity of SOD in crude extract of DR1.

3.3.6 Effect of Mn^{2+} and Cd^{2+} on survival after UV exposure

To analyze if UV damage could provide any cross resistance to the cells recovering in presence of Cd^{2+}, DR1 cells were exposed to UV and subsequently plated on Cd^{2+} containing plates. An enhanced survival of the cells recovering in presence of Cd^{2+}, amounting to about 1.37 times of the control, was observed which can be attributed to induction of the *uvr* genes and *rec*A for the repair of UV induced damage (Fig. 3. 19). Effect of Cd^{2+}on *rec*A^- mutant, TNK106 was examined. The *rec*A^- disruptant mutant, TNK106, proved to be more sensitive to Cd^{2+}while in presence of Mn^{2+}, Cd^{2+} toxicity seemed not to be significantly affected (Fig. 3.20). The *rec*A mutant demonstrated a D_{50} value of 0.925μg/ml (5.04 μM), while the wild type strain has a D_{50} value of 3.9 μg/ml (21 μM) which amounted to 4 fold sensitivity of the rec A mutant (Fig. 3.21). Direct role of homologous recombination for removal of UV photoproducts from genomic DNA DR1 has been reported (Tanaka et al., 2005). Our results are in accordance to the fact that UV induced recA in DR1 can facilitate enhanced recovery in presence of Cd^{2+}. The cells growing on TGY medium accumulate high levels of metabolic intermediates, which are necessary for DNA repair. The induction of an EMP pathway by Mn^{2+} depletes G-6-P and thus the precursors for the nucleotide synthesis. (Zhang et al., 2003). This explains the increase in UV sensitivities of TGY+Mn^{2+} recovered cells.

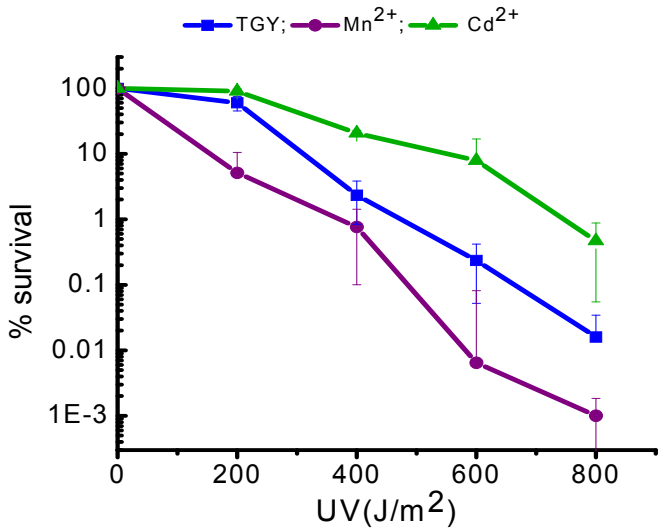


Fig. 3.19: Effect of Cd^{2+} and Mn^{2+} on recovery of DR1 from UV radiation. The recovery was observed on TGY or TGY+100µM Mn^{2+} or TGY+ 2.5 µM Cd^{2+}

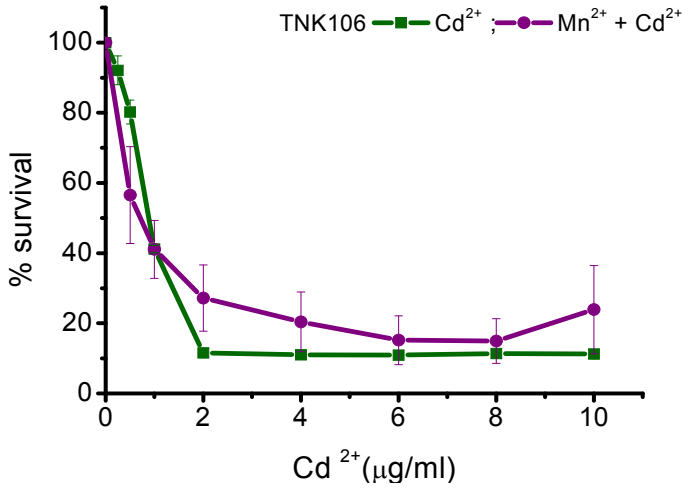


Fig. 3.20: Effect of Cd^{2+} on *rec* A^- mutant of DR1 in presence and absence of 100 µM Mn^{2+}

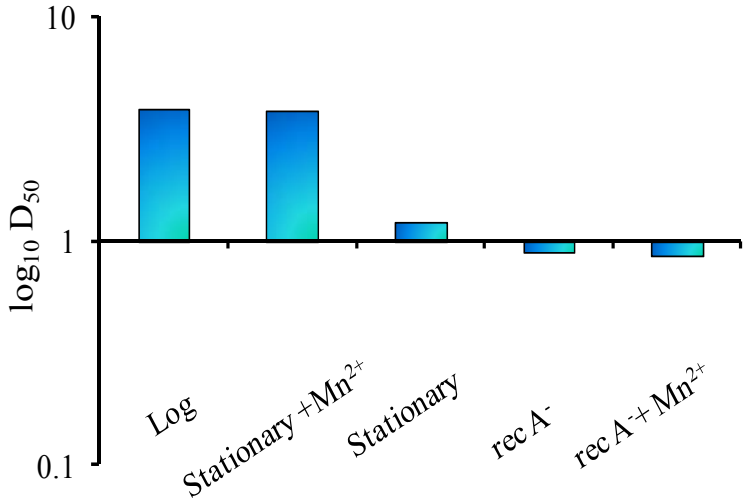


Fig. 3.21: A comparative analysis of Cd^{2+} D_{50} for the wild type DR1 grown under different growth phases and the *rec*A- mutant. The influence of Mn^{2+} on D_{50} for Cd^{2+} has been compared for both the wild type and the *rec*A^- mutant.

To assay if Cd^{2+} affects the expression of recA in DR1, we created translational fusion of the *rec*A promoter of DR1 to *lac* Z reporter gene by the PCR amplification of *rec* A promoter (p*rec*A) of DR1 as 400 bp fragment from genomic DNA using specific primers (Fig. 3.22 A), and cloning of the same in pTZ57R/T.

The pTZ57R/T clones were confirmed using promoter specific restriction enzymes, *Spe*I and *Bam*HI (Fig. 3.22 B) and amplification using promoter specific primers (Fig. 3.22 C). *gro*EL promoter from pRADZ3 was excised as a *Spe*I and *Bam*HI fragment followed by sub-cloning of recA promoter in *Spe*I and *Bam*HI site of pRADZ3. The clones obtained in *E.coli* DH5α were confirmed using *Spe*I and *Bam*HI and PCR amplification of the cloned fragment (Fig. 3.23). The confirmed plasmid, prec A - pRAD Z3 (Fig. 3.24) was transformed in DR1 to obtain DR1 (prec A - pRAD 4Z3).

The effect of Cd^{2+} appears immediately as evident from increased activity at the onset. There was a dose dependent increase of the *lac*Z activity; however the activity decreased with prolonged incubation (Fig. 3.25). Min et al., (1999), created a *rec*A operator-promoter *luxCDABE* fusion to demonstrate the induction of *rec*A activity on exposure to several mutagenic agents in *E.coli*. The authors demonstrated the dose dependent enhancement of the *rec*A activity on Cd^{2+} exposure. The *rec*A promoter was upregulated in presence of Cd^{2+} in DR1 however prolonged incubation

diminished the effect. The results are in agreement that Cd^{2+} induces the *rec*A activity indicating the possibility of the DNA damage although it cannot be stated if the effect is direct or indirect.

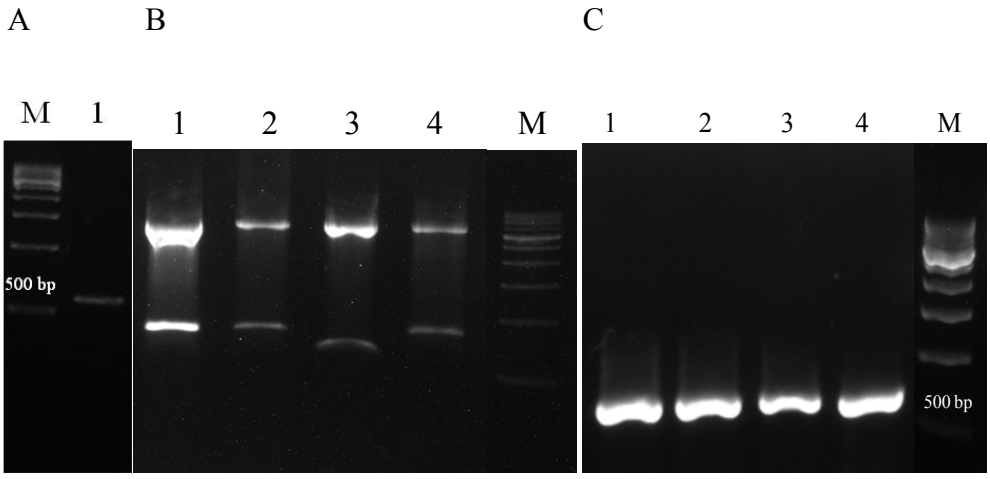


Fig. 3.22: Amplification and clone confirmation of the pTZ57R/T- A) amplification of precA from DR1 genomic DNA; B) Clone confirmation using *Spe*I and *Bam*HI; C) PCR amplification from the clones. Lane 1: 8T-precA; Lane 2: 9T-precA; Lane 3: 16T-precA; Lane 4:20T-precA; M: 500 bp marker. Clone 8T-precA was used for further sub-cloning in pRADZ3.

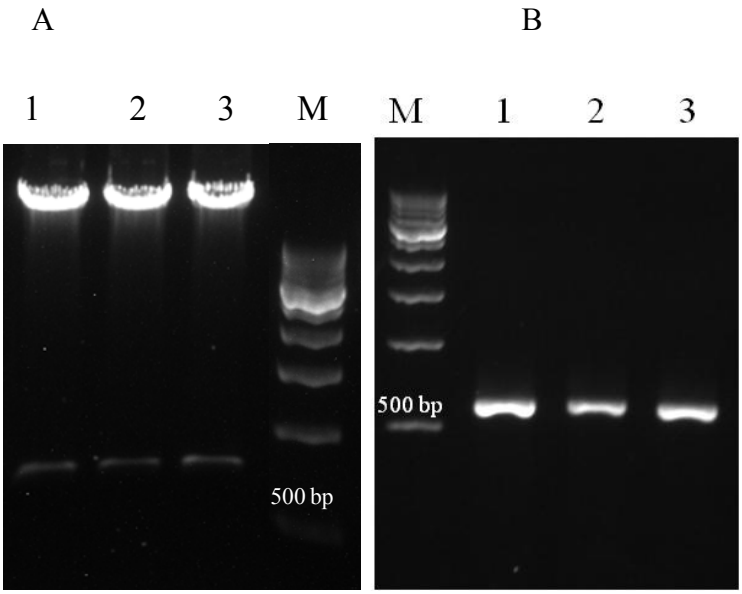


Fig. 3.23: Clone confirmation of the prec A - pRAD Z3clones using. A) *Spe*I and *Bam*HI; B) PCR amplification. Lane 1: precA- 4Z3-; Lane 2: precA-7Z3; Lane 3: precA- 10Z3; M: 500bp marker. precA- 4Z3 was selected for transformation in DR1.

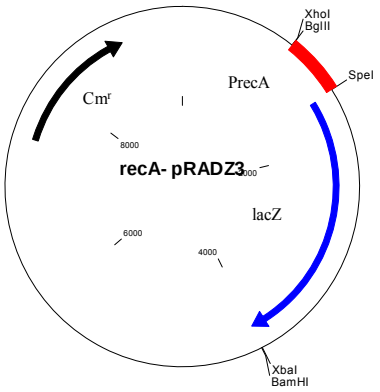


Fig. 3.24: Vector map of prec A -pRAD Z3.

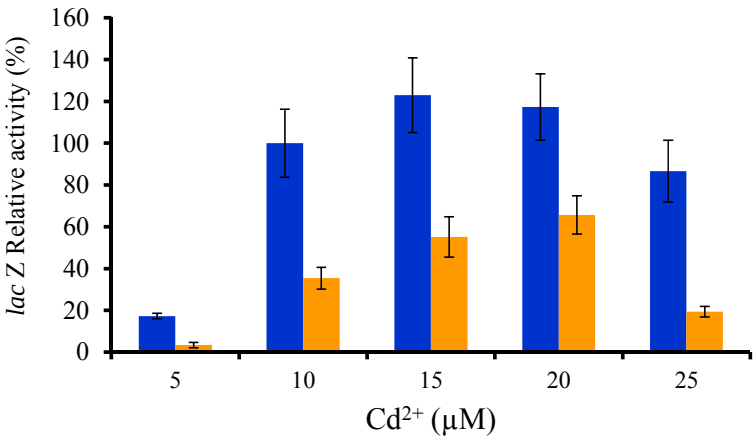


Fig. 3.25: Effect of Cd^{2+} on *rec*A promoter. Activity was assayed by using prec A- lac Z reporter in DR1. The relative activity is measured by normalizing the *lac* Z activity against the control. The blue bars indicate 1.5h and yellow bars indicate 3h post recovery.

Cd^{2+} is known to cause a single stranded break (Mitra and Bernstein, 1978) which was further affirmed by the proteomic analysis exhibiting the upregulation of *xth*A endonuclease in *E.coli* (Ferianc et al., 1998). Trancriptome analysis of *E.coli* exposed toCd^{2+} exhibit upregulation of *recA*, *dnaN, dinJ*, and *uvrB* suggesting that the main repair pathway activated by Cd^{2+} is the nucleotide excision repair system, which typically responds to UV and is characterized by involvement of the *uvr* system (Wang et al., 2005). Similar gene expression has been observed in DR1 emphasing

the role of recombinational and base excision repair for the cells exposed to Cd^{2+}(Joe et al., 2011).

3.3.7 Effect of Cd^{2+} on transcriptional regulator, PprI and PprM in DR1

PprI is a transcriptional regulator in DR1 that regulate the expression of *rec*A and is highly expressed in irradiated cells of DR1 (Earl et al., 2002; Ohba et al., 2005). Recently it was observed that PprI acts through PprM (Ohba et al., 2009) that enhances the expression of PprA, which in turn activates catalase (Kota and Misra, 2006) however PprM has no effect on the activity of recA (Fig. 3.26). The effect of Cd^{2+} toxicity on *ppr*I^- and *ppr*M^- mutant (Fig. 3.27), shows *ppr*I^- mutant exhibited increased sensitivity to Cd^{2+} with a D_{50} of 0.49µg/ml (2.6 µM) which is less than *rec*A^- mutant or *sod*A^- mutant. As shown in Fig. 3.26, PprI regulates both catalase, through PprM, and RecA further affirming the fact that Cd^{2+} affects both ROS combating enzymes as well as DNA repairing enzymes.

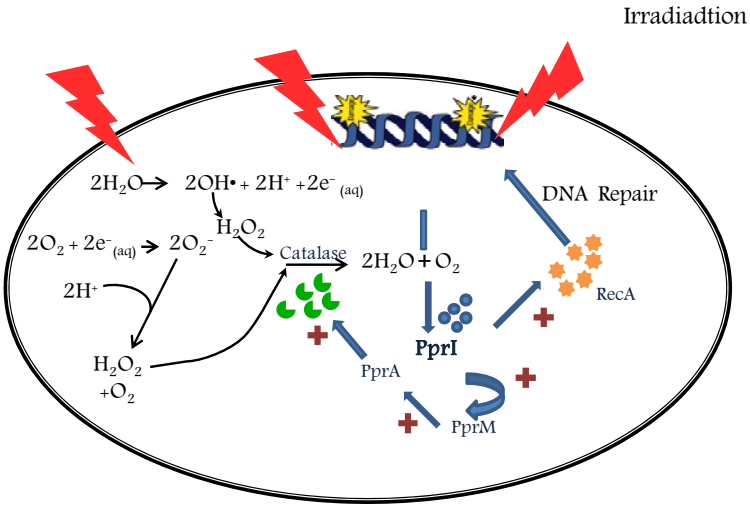


Fig. 3.26: PprI mediated control of *rec*A and catalase activity in DR1, (+) indicates the positive regulation of the genes (compiled from Slade and Radman, 2011).

3.3.8 Protein profiling in response to Cd^{2+} in DR1

The apparent change in response to Cd^{2+} stress during stationary phase in DR1 was appalling as it is considered that most of the bacterial cultures tend to be resistant to external stress in stationary phase due to inherent upregulation of several stress regulons during stationary phase (Nystrom, 2004; Ishihama et al., 1997).

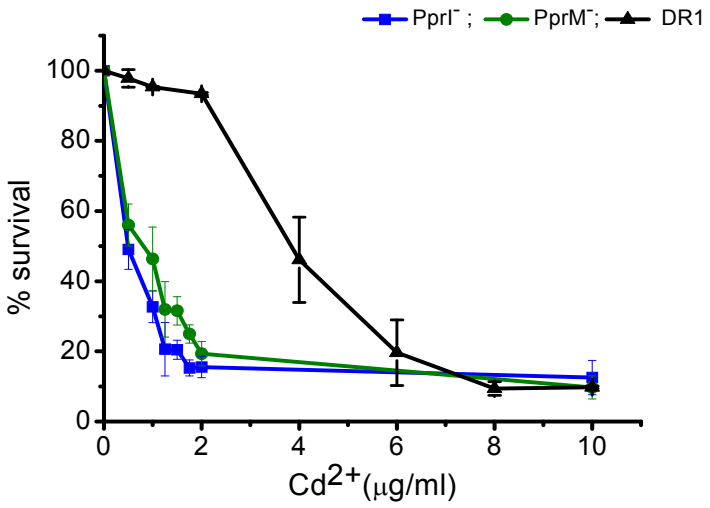


Fig. 3.27: Cd^{2+} toxicity to $PprI^-$ and $PprM^-$ mutant of DR1.

Sukhi et al., (2009) reported the differential radiation resistance in DR1 influenced by the growth state, which was attributed to the lack of stationary phase specific sigma factor, σ^S. To gain further insight into the mechanism that may operate for Cd^{2+} sensitivity during the stationary phase protein profiles of the Cd^{2+}-stressed cultures grown under different growth phases was analysed. Cd^{2+} affected the proteome wherein a large majority of proteins were either repressed or had undergone degradation. In particular, absence of proteins in the range of 29 kDa and 14 kDa indicate degradation of the proteins while those in the range of 68-43 kDa appear to have repressed with respect to other conditions, stationary phase, log phase and Mn^{2+} amended to the log phase cultures, used in the study (Fig. 3.28). On the contrary several proteins appeared to be strongly upregulated in case of stationary phase cultures as opposed to log phase cultures. Mn^{2+} exposed culture appeared similar except for a single band > 18.4 kDa appears to be strongly upregulated (Fig. 3.28). Analysis of 2-D gels using Melanie 7 software taking log phase treated cells as the reference gel showed that, of the 41 spots detected by the software in the log phase protein sample, 32 % were common with other conditions while Cd^{2+} treated cells have surprisingly only a single protein matching with reference log phase cells of DR1. Stationary phase cells and Mn^{2+} treated cells showed 23 % and 36 % respectively similarity to the log phase proteome (Fig. 3.29).

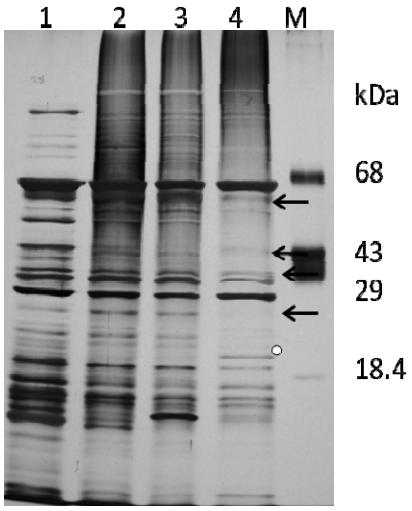


Fig. 3.28: SDS-PAGE analysis of total cellular proteins from DR1 treated with Cd^{2+} under different growth conditions, 1. Stationary phase; 2. Log phase; 3. Log + 100 µ M Mn^{2+}; 4. Log + 2.5 µ M Cd^{2+}

In accordance to the SDS-PAGE profile, Cd^{2+} treated cells show a decline in the number of proteins spots. Spot detected in Fig. 3.30 panel 1 was completely degraded under stress condition, while in Fig.3.30 panel 2 the spot in log phase was repressed in all stress conditions. Mn^{2+} and Cd^{2+} affected proteins showed appreciable similarity as reflected in Fig. 3.30 panel 3. Some common proteins were detected under Mn^{2+}, Cd^{2+} induction and stationary phase cultures as depicted in Fig. 3.30 panel 4.

There was overall large amount of similarity amongst the proteins expressed during stationary phase, Mn^{2+} induced and Cd^{2+} induced cultures of DR1, therefore it can be concluded that exogenous Mn^{2+} can also exert stressful conditions in DR1. No spots were detected exclusively for both stationary phase cultures and Cd^{2+} affected cells indicating that DR1 has more generalised response to combat the Cd^{2+} stress and no unique mechanism to combat Cd^{2+}. Further identification of these proteins may incur insight into the exact mechanism to handle stress in DR1. Heat shock proteins, Mn^{2+} superoxide dismutase, ClpB protease form a common response proteins expressed under Cd^{2+} in *Campylobacter jejuni* (Kaakoush et al., 2008) and in *Corynebacterium glutamicum* (Fanous et al., 2008). These observations indicate that Cd^{2+} does induce oxidative stress and thereby damaging proteins which is affirmed by the induction of heat shock proteins as well as protease such as ClpB

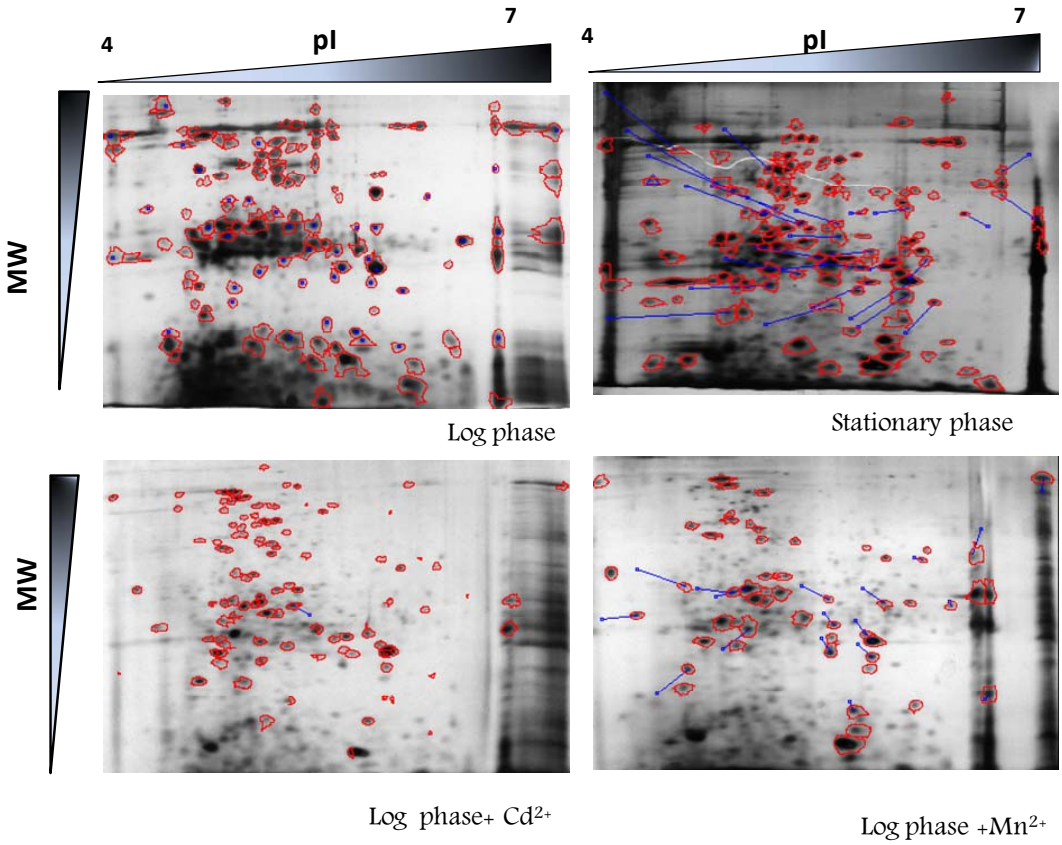


Fig. 3.29: 2D gel electrophoresis of DR1 under different growth conditions. The analysis was done using Melanie 7. The spots marked in red were used for analysis while the blue vectors represent the common proteins detected with log phase as reference gel.

3.3.9 Cd^{2+} and Mn^{2+} binding proteome of DR1

The metallo-proteome is defined as the set of proteins that have metal-binding capacity by being metalloproteins or having metal-binding sites. A metalloproteome may include proteins that are unique for a metal as well as those that may be shared with some metal. The proteins that bind metal are more susceptible to metal catalysed oxidative damage. Affinities of proteins for metals have a tendency to follow a universal order of preference, which for essential divalent metals is the Irving–Williams series, given as: Mg^{2+} and Ca^{2+} (weakest binding) < Mn^{2+}< Fe^{2+}< Co^{2+}< Ni^{2+}< Cu^{2+}> Zn^{2+}. By restricting the effective concentration of the competitive metals at the top of the Irving–Williams series, metal-binding sites remain available to less-competitive, weak binding inorganic ions (Waldron and Robinson, 2009). DR1 accumulates large amount of Mn^{2+} intracellularly (Daly et al., 2004; Ghosal et al., 2005),

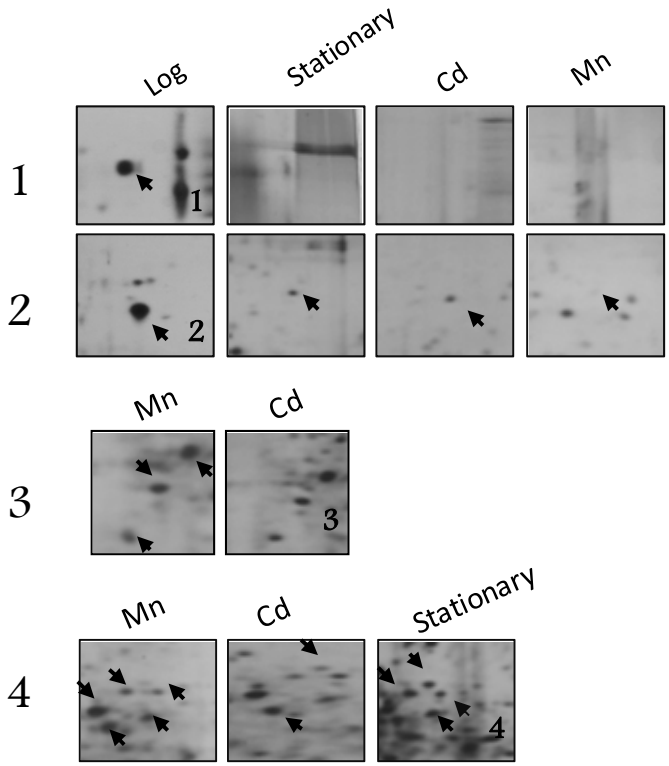


Fig. 3.30: Proteins differentially expressed under different growth conditions in DR1 Panel 1). Protein spots (indicated by arrow) differentially expressed in log phase but not present in other conditions tested; 2) Indicates protein that are strongly repressed in log phase but repressed under all other growth conditions; 3) Proteins expressed both in presence of Mn^{2+} as well as Cd^{2+}; 4) are Proteins that commonly expressed under stationary phase , Mn^{2+} and Cd^{2}

This is supported by the fact that several enzymes of DR1 employ Mn^{2+} as co-factor such as the class II fructose-1,6-bisphosphate aldolase uses Mn^{2+} instead of Zn^{2+} as cofactor (Zhang et al., 2006), Proteins differentially expressed under different growth conditions in DR1 employ Mn^{2+} as co-factor as in RNA ligase (Martins and Shuman, 2004), (di)adenosine polyphosphate hydrolase (Fisher et al., 2006),UV endonuclease β (Evans and Mosley, 1995), DNA pol. X (Blasius et al., 2006), Mn-SOD (Juan et al., 1991), NAD dependent DNA ligase (Blasius et al., 2006). Assuming that Cd^{2+} may compete with Mn^{2+} and other essential metals for binding with proteins, it was of interest to study the Cd^{2+} and Mn^{2+} binding proteome of DR1. To study the metallo-proteome IMAC was employed.

As seen in the Fig. 3.31 there is appreciable similarity between the proteins that are bound to Mn^{2+} and Cd^{2+} under all the conditions tested while no proteins were retained on the control column, indicating the possibility of several Mn^{2+} interacting protein that can bind Cd^{2+} as well. Apart from the common proteome represented

under conditions listed in Fig. 3.31 the proteins represented between 49 and 23 kDa formed a unique fingerprint of the stationary phase culture not represented in any other proteome. Of the stationary phase proteome a band > 97 kDa was found only in the eluate of Cd^{2+} binding proteome while a band of 29 kDa was found to be unique to log phase Mn^{2+} binding proteome not represented in the respective Cd^{2+} proteome. Mn^{2+} induced proteome also reflected unique proteins binding to Cd^{2+} only and not Mn^{2+} in the range of 66 kDa and 43 kDa, surprisingly there was no observable difference reflected in Mn^{2+} and Cd^{2+} binding proteins in Cd^{2+} induced proteins. Sequence and consequent structural analysis shall be able to consolidate our results.

In-silico analysis of the band pattern from the individual proteome was performed which revealed the similarity between Cd^{2+} induced, Mn^{2+} binding proteins as well as Cd^{2+} binding proteins to the stationary phase Mn^{2+} binding proteome (Fig.3.32). This justifies the fact that there could be several targets for Cd^{2+} during the stationary phase which possibly explains the sensitivity of the stationary phase cultures to Cd^{2+} than the log phase.

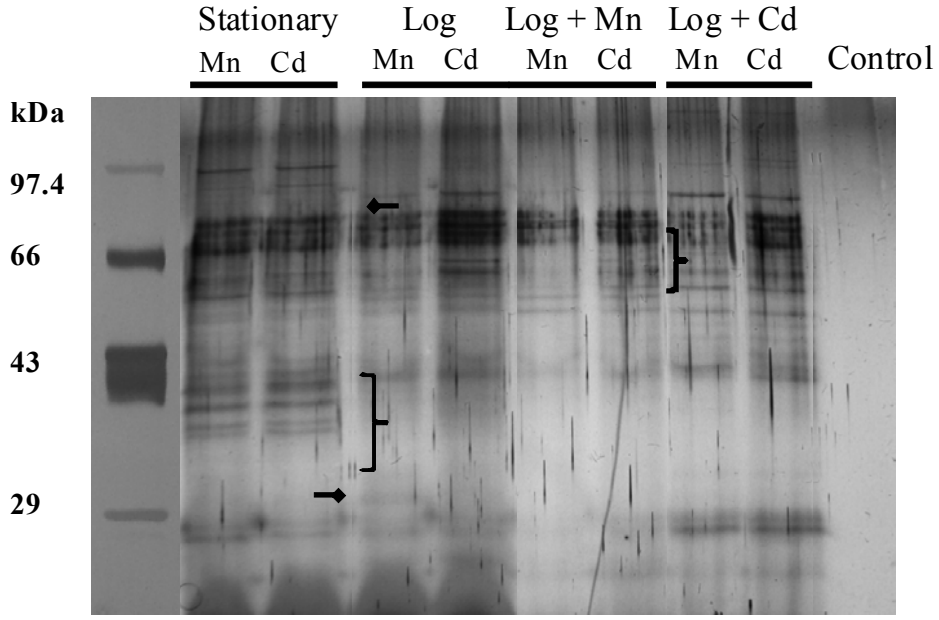


Fig. 3.31: SDS PAGE of Mn^{2+} and Cd^{2+} binding proteins in DR1 obtained by IMAC.

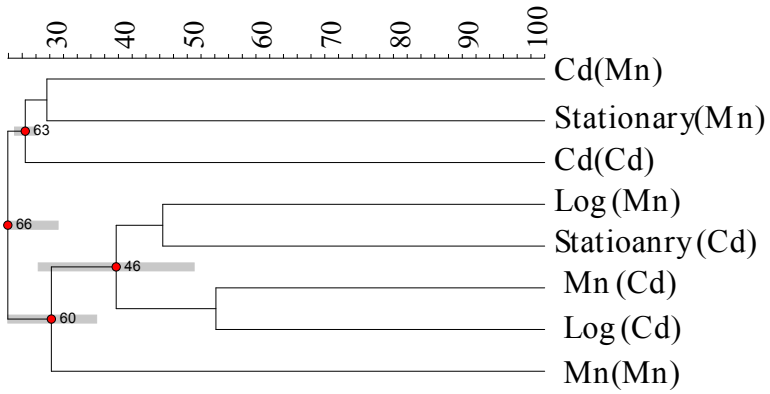


Fig. 3.32: Dendrogram obtained from *in silico* analysis of the proteins represented in Fig. 3.31. UPGMA was employed to generate the dendrogram. The values at the nodes indicate the % similarity.

A comparison between the metallo-proteome of specific metal under different growth conditions reveal that proteins binding to Mn^{2+} column under all growth condition (Fig. 3.33 (A)) revealed the presence of bands indicated as band 1 (97.4 kDa), band 2 (>97.4 kDa), band 3 (43 k Da) and a doublet band 4 (>43 kDa) unique to stationary phase cultures while band 5 was only represented in the Cd^{2+} induced cultures. Presence of common Mn^{2+} biding proteome reflect a large number of proteins are capable of binding to Mn^{2+} and might be essential for the normal growth. However there were only two unique bands binding to the Cd^{2+} column each belonging to Mn^{2+} and Cd^{2+} induced cultures of DR1 (Fig. 3.33(B)).

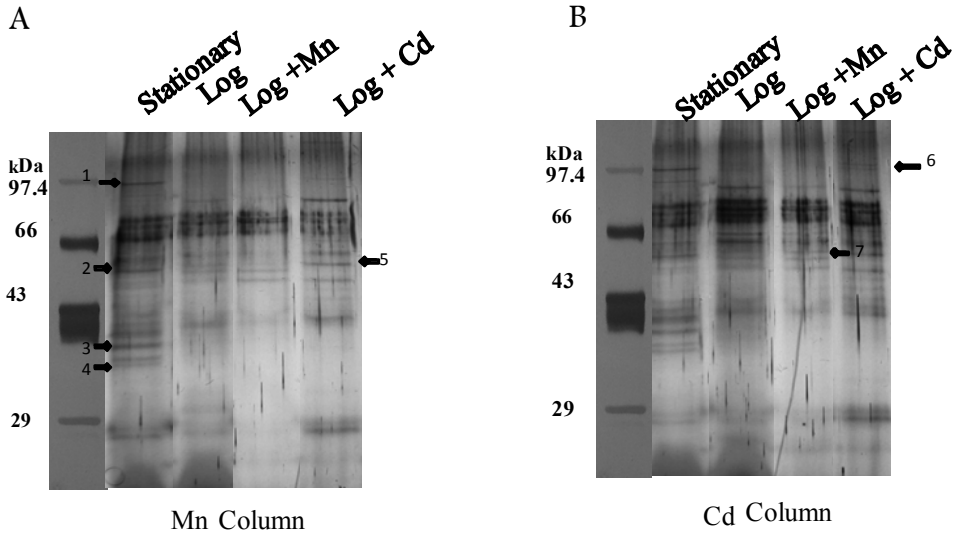


Fig. 3.33: IMAC purification of A) Mn^{2+} binding proteome of the DR1 cells; B) Cd^{2+} binding proteome of DR1 cells. Arrows indicate unique bands discussed in the text.

3.4 Conclusion

DR1 is considered as the most potential candidate for the bioremediation of nuclear waste sites. DR1 and other radiation resistant organisms employed in the study were found to be sensitive to Hg^{2+} and Cd^{2+}. Amongst the radiation resistant organisms examined for Hg^{2+} toxicity Grk2 was found to be most resistant, while Grk4 was most resistant to Cd^{2+}. DR1 exhibited comparatively higher level of resistance for Cr^{6+}.

Cd^{2+} toxicity in DR1 was affected by growth phase with stationary phase cells being more sensitive than the exponential phase. Stationary phase of DR1 cells have been earlier shown to be more radiation sensitive as opposed to the exponential phase cultures (Sukhi et al., 2009). The toxicity of stationary phase cells was ameliorated by addition of exogenous Mn^{2+}, indicating the possible competitive role of Mn^{2+}

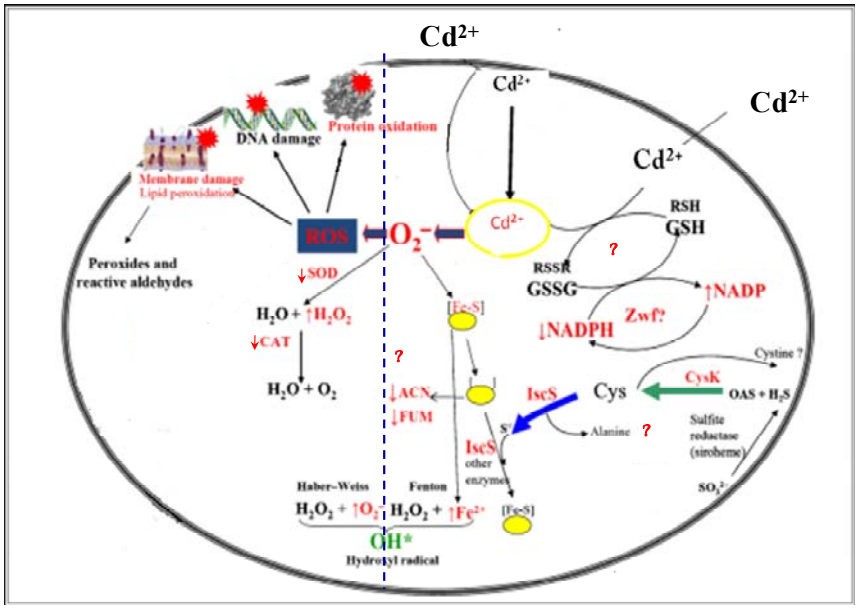


Fig. 3.34: Effect of Cd^{2+} on different ROS pathways in DR1. The pathways marked with question mark, right of the demarcated line, may play a possible role in tolerance towards Cd^{2+} but need to be addressed.

Cd^{2+} is known to elicit oxidative stress most likely indirectly by replacing Fe, from Fe-S clusters, free Fe is capable of generating and propagating ROS by promoting Fenton's chemistry. In our studies Cd^{2+} was demonstrated to elicit ROS response leading to oxidative damage to proteins and lipid peroxidation. Diminution in SOD and catalase activity with concomitant increase in ROS explains Cd^{2+} toxicity in DR1. The recA-mutant was found to be sensitive to Cd^{2+} as compared to the wild type. Additionally,

dose-dependent response of recA-*lac* Z reporter assay in DR1 indicates that Cd^{2+} is capable of asserting DNA damage in DR1. A concerted effect of increase in ROS, inactivation of the ROS combative enzymes and DNA damage further aggravates the Cd^{2+} stress in DR1. Due to high Requirement of high intracellular Mn^{2+} by DR1 implies existence of several proteins that may interact with Mn^{2+} which may act as potential targets for Cd^{2+} toxicity. This was affirmed by the presence of several common proteins that bind both Mn^{2+} and Cd^{2+} under varying growth conditions. Further studies needs to be undertaken to reveal the identity of the Cd^{2+} binding proteins to understand the exact mechanism of Cd^{2+} toxicity in DR1. Fig. 3.34 summarises the observations in this study.

Chapter 4: Cloning and expression of metallothionein genes in Deinococcus radiodurans R1

If a man will begin with certainties, he shall end in doubts; but if he will be content to begin with doubts he shall end in certainties- Sir Francis Bacon

4.1 Introduction

Many metal ions are essential as trace elements but at higher concentrations they become toxic. Heavy metals are difficult to remove from the environment and unlike many other pollutants cannot be chemically or biologically degraded and are ultimately indestructible. Microorganisms could be used to clean up metal contamination by removing metals from contaminated waste by sequestering metals from soils and sediments, or solubilizing metals to facilitate their extraction. Detoxification of metals by the formation of complexes is a strategy used by most eukaryotes (Nies, 1999).

Metallothioneins (MTs) are low molecular weight (6–7 kDa), cysteine-rich proteins found in animals, higher plants, eukaryotic microorganisms and some prokaryotes. They are divided into three different classes on the basis of their cysteine content and structure (Cobbett and Goldsbrough, 2002). The Cys-Cys, Cys-X-Cys and Cys-X-X-Cys motifs (in which X denotes any amino acid) are characteristic and invariant for MTs. The first prokaryotic MT to be identified is from cyanobacterial strains of the genus *Synechococcus* which is encoded by the *smtA* gene, contains fewer cysteine residues than mammalian MTs (Huckle et al., 1993). Deletion of the *smt* locus reduces Zn/Cd tolerance (Turner et al., 1993). The *smt* locus includes *smt*A, which encodes a class II MT (Olafson et al., 1988) and a divergently transcribed gene *smt*B which encodes a repressor of *smt*A transcription (Huckle et al., 1993). SmtA has at least three distinct metal binding sites coordinates to three Zn^{2+} ions via eight Cys residues. Two metal sites contain exclusively Cys-thiolate ligands, whereas the third contains both Cys-thiolate and His- imidazole ligands (Blindauer et al., 2001; Blindauer et al., 2002).

Phytochelatins (PCs) are short, cysteine-rich peptides with the general structure $(\gamma Glu\text{-}Cys)_nGly$ (n 4 2–11). PCs offer many advantages over MTs due to their unique structural characteristics, particularly the continuously repeating γGlu-Cys units. The presence of a γ bond between glutamic acid and cysteine in PCs indicates that these peptides must be synthesized enzymatically. An attractive alternative strategy is to develop organisms harbouring synthetic genes encoding protein analogs of PC with the general structure $(Glu\text{-}Cys)_{nGly}$ (ECs). These peptides differ from PCs because the peptide bond between glutamic acid and cysteine is not the γ bond since synthetic phytochelatin are synthesised by the ribosomal machinery (Malin and Bulow, 2001).

Bae et al., (2000) demonstrated the efficacy of the synthetic phytochelatin in chelating Cd^{2+}.

Radioactive waste sites are a concoction of several hydrocarbons such as trichloroethylene, toluene and xylene apart from the radioactive metals such as uranium, plutonium, caesium and non-radioactive heavy metals such as cadmium, mercury, lead and chromium. Due to the prohibitive cost of cleaning up of the nuclear waste, bioremediation is an attractive alternative for the clean-up of the nuclear waste sites. However there are certain inherent disadvantages of using *D. radiodurans* R1 (DR1). It has been found that nutrient conditions have a profound effect on the survival and growth ofDR1duringchronic exposure to irradiation (Venkateswaran et al., 2000). Ruggiero et al., (2005) have demonstrated that the concentrations of metals that inhibit DR1 growth are lower than the concentration inhibitory to other bacteria.

The fact that DR1 is not extraordinary in its tolerance to metals and proximate radionuclides is not surprising. DR1 can grow while exposed to exceptionally high dose of 6000 rads h^{-1} (60 Gy h^{-1}) γ radiation from an external source. Metal toxicity is generally related to cell penetration and subsequent damage to the cellular machinery (Silver, 1998; Sarkar, 2002) while DR1's radiation resistance is attributed to fast and efficient DNA repair mechanisms (Battista, 1997; Battista et al., 2000; Fredrickson et al., 2000), its radiation resistance is unlikely to correlate with its actinide or heavy metal resistance except in cases where the actinide or the metal directly catalyses DNA damage. The expression of metal reduction or resistance genes or expansion of its metal reduction abilities could augment DR1's functionality (Fredrickson et al., 2000).

Deinococcal strains demonstrate exceptional sensitivity to Cd^{2+} (Ruggiero et al., 2005; this work, Chapter 3). Immobilization by intracellular binding to metallothionein is an attractive alternative to reduce the toxicity of Cd^{2+} and other heavy metals in deinococci. DR1 has been reported to be transformed for varied bioremediative processes (Lange et al., 1998; Brim et al., 2000; Brim et al., 2006; Appukutan et al., 2006). Albeit the success of the recombinant strains at the lab scale their field application has never been demonstrated. Construction of recombinant strains of DR1 that can survive high metal concentrations at the radioactive waste sites and subsequent co-transformation of such strains with metabolic genes for mineralization of toxic hydrocarbon and metal can enhance the applicability of DR1 for bioremediation at the nuclear waste sites. Quin et al., (2006) demonstrated the

expression of MerR metal binding domain for enhanced metal sequestration in DR1. Considering the bioremediative potential of DR1, this work deals with the intracellular expression of metallothioneins (both synthetic and natural) in DR1 to expand the metal tolerance for its better applicability at the nuclear waste sites.

4.2 Material and Methods

4.2.1 Bacterial strains, and plasmids

The bacterial strains and plasmids used in this study as described in Table 4.1.

Table 4.1 Strains and Plasmids used in this study

Strain or Plasmid	Relevant information	Source/Ref.
***E. coli* DH5α**	*supE44* D*lacU(*f*80lacZDM15) hsdR17 recA1 endA1 gyrA96 thi 1 relA1*	Laboratory stock
***E. coli* BL-21(DE3)**	F-*omp*T *gal*(*dcm*) (*lon*) *hsdS*$_B$ (r_B^- m_B^-an *E.coli* B strain)with DE3,a λ prophage carrying the T7 polymerase gene	Lab. stock (Sambrook & Russell, 2001)
***D. radiodurans* R1 ATCC13939**	Wild type	Prof. Mary Lidstrom, University of Washington, Seattle, USA.
DR1 (pradZ3)	DR1 harbouring pRADZ3	This study
DR1 (pRAD-*EC*)	DR1 harbouring pRAD-*EC*	This study
DR1 (pRAD-*smt* A)	DR1 harbouring pRAD-*smt*A	This study
	Plasmids	
pTZ57R/T	T-vector for the cloning of PCR products Ap^r	MBI Fermentas, Germany
pRADZ3	Shuttle vector in *E. coli* and DR1, *lacZ* fragment of pMUTIN2mcs, putative R1 *groESL* promoter, Ap^r Cm^r	Prof. Mary Lidstrom. University of Washington, Seattle, USA Meima and Lidstrom, 2000.
pMHNR1.1	pet 29(a) carrying the cloned *smt* A gene of *Synechococcus* PCC 7942	Prof. Nigel.J.Robinson. University of Durham, Durham, United Kingdom. Blindauer et al., (2001)
pTZ57R 6st	pTZ57R/T vector carrying the in vitro synthesized *ec*20	This study
pRAD-*EC*	pRADZ3 containing the *ec*20	This study
pRAD-*smt*A	pRADZ3 containing the *smt*A	This study

4.2.2 Chemicals and enzymes

All chemicals used were of analytical grade and, unless indicated otherwise, were obtained from Hi-media Pvt. Ltd., Mumbai, India or Merck, India. Enzymes for molecular biology were purchased from MBI Fermentas, Germany or Bangalore genei Pvt. Ltd., Bangalore, India, and used according to the supplier.

4.2.3 Media and growth conditions

D. radiodurans R1 (DR1) was routinely grown at 30°C in TGY broth (0.5 % tryptone; 0.1 % glucose; 0.3 % yeast extract). DR1 (pRAD-*EC*) and DR1 (pRAD-*smt* A) were cultivated in TGY supplemented with 3 µg/ml chloramphenicol. *E. coli* strains were grown at 37 °C in Luria–Bertani (LB) broth or on LB agar supplemented with 100 µg/ml ampicillin for strains harbouring pTZ7R/T, while pMHNR1.1 and pRADZ3 transformants were cultivated with 50 µg/ml ampicillin.

4.2.4 Synthesis and PCR amplification of the ec20

The two oligonucleotides EC 1 and EC 2 that were used for the synthesis of the synthetic gene *ec20* were obtained from MWG-Biotech AG. Their sequences are as follows

EC1

5’TAGCTT***CCCGGG***AATGTGAATGTGAATGTGAATGTGAATGTGAATGTGA
ATGTGAGTGTGAATGTGAGTGCGAATGCGAA3’

EC2

5’TGCCG***TCTAGA***TTAACCACATTCACATTCACATTCACATTCACATTCACA
TTCGCATTCACATTCGCATTCGCATTCGCACTC3’

The sequence in bold faced, italics represents the recognition site of *Xma*I and *Xba*I in EC1 and EC2 respectively. The under- lined region represents the region of overlap. Following primers are used for the amplification of the synthetic gene

Forward Primer ECF:

5’GCT***ACTAGT***AGGAGGACCCCACATGACCCGGGAAGAATGTGAATGT 3′

Reverse Primer ECR:

5’-TGCCG***TCTAGA***TTAACCA-3’

The sequence in bold faced, italics represents the recognition site of *Spe* and *Xba*I in ECF and ECR respectively the under lined sequence represents the ribosome binding site.

*ec*20 was synthesized using the PCR based strategy wherein EC1 and EC2 were combined at a concentration of 0.3 ng each in a system of 30 µl comprising of 3µl of 10X reaction buffer,1µl of 10 mM dNTPs and 1.5 u of *Taq.* DNA polymerase. The PCR cycling conditions for the synthesis of the *ec*20 were initial denaturation at 95 ˚C for 3min followed by ten cycles each comprising of denaturation at 95 ˚C for 30s, annealing at 41 ˚C for 30s and extension at 72 ˚C for 45 s. After 10 cycles template for PCR amplification were generated. At the end of the 10 cycles ECF and ECR were supplemented at a concentration of 0.3µM and the PCR was continued for 30 cycles with the PCR cycles consisting of denaturation of 95˚C for 30s, annealing at 46˚C for 30s and extension at 72 ˚C for 45 s with final extension at 72 ˚C for 10 min.

*ec*20 was synthesized using the PCR based strategy wherein EC1 and EC2 were combined at a concentration of 0.3 ng each in a system of 30 µl comprising of 3µl of 10X reaction buffer, 1 µl of 10mM dNTPs containing 2.5 mM each and 1.5 U of *Taq.* DNA polymerase. The PCR cycling conditions for the synthesis of the *ec*20 were initial denaturation at 95˚C for 3min followed by ten cycles each comprising of denaturation at 95˚C for 30s, annealing at 41˚C for 30s and extension at 72 ˚C for 45 s. This would result in the generation of the double stranded *ec*20 molecules by overlap extension of EC1 and EC2 which were to be subsequently used as template for PCR amplification with ECF and ECR. At the end of the 10 cycles ECF and ECR were supplemented at a concentration of 0.3µM and the PCR was continued for 30 cycles with the PCR cycles consisting of denaturation of 95 ˚C for 30 s, annealing at 46 ˚C for 30 s and extension at 72 ˚C for 45 s with final extension at 72 ˚C for 10 min.

4.2.5 PCR amplification of smtA

The *smt*A gene, encoding the metallothionein from *Synechococcus* PCC 7942, was PCR amplified from the plasmid pMHNR1.1 using the primers

smtA F: CT***ACTAGT***AGGAGGACCCCACATGACATGACCTCA

smtA R: GT***GGATCC***ACTACAGTTGCAGCCGGTGTG

The sequence in bold faced and italics denote the *Spe*I and *Bam*HI sites in smtA F and smtA R primers, respectively, while the under-lined sequence is the RBS. An appropriate dilution of pMHNR1.1 DNA was combined in a system of 30µl comprising of 3 µl of 10X reaction buffer, 1µl of 10 mM dNTPs, 1.5 U of *Taq.* DNA polymerase and 0.3 µM each of the forward and reverse primers. The PCR cycling conditions were: initial denaturation at 95 ˚C for 3min followed by 30 cycles each

comprising of denaturation at 95 ˚C for 30 s, annealing at 58 ˚C for 45s and extension at 72 ˚C for 30 s and a final extension of 72 ˚C for 10 min. The products of the PCR were analysed on 2 % agarose gel.

4.2.6 DNA manipulations

a) Plasmid extraction

Miniscale plasmid DNA preparations from *E.coli,* restriction digestion and ligation were done by standard protocols described by Sambrook and Russell, (2001). Transformation of *E. coli* strains was done using $CaCl_2$ method (Sambrook and Russell, 2001).

b) Cloning in T-vector

The PCR products obtained were purified by home- made spin columns (Wang and Rossman, 1994) and cloned in pT57R/T following manufacturer's (MBI Fermentas, Germany) instructions, and transformed in *E. coli* DH5α using $CaCl_2$ method (Sambrook and Russell, 2001).The clones were confirmed by digestion with *Eco*RI and *BamH*I and PCR amplification. The PCR cycling conditions were the same as described earlier.

c) Construction of pRAD-EC

The plasmid from T-vector clone, 6ST was digested with *Spe*I and *Xba*I and the insert was purified. The purified *ec* 20 gene was cloned into the *Spe*I, *Xba*I site of purified and dephoshorylated vector, pRADZ3. The ligated product was transformed in *E. coli* DH5α and selected on 50 µg/ml ampicillin. The clones were confirmed by PCR as described above and the products were analysed on 2 % gel. *Spe*I and *Xba*I digest of the clones were resolved on 10 % acrylamide gel with subsequent silver staining to confirm the presence of insert.

d) Construction of pRAD- smtA

*Spe*I and *Bam*HI digested amplified fragment of *smt*A were cloned in *Spe*I and *Bam*HI digested purified pRADZ4. The ligated product was transformed in *E.coli* DH5α and selected on 50 µg/ml ampicillin. The clones were confirmed by PCR as described above and the products were analysed on 2 % gel. *Spe*I and *Xba*I digest of the clones were resolved on 10 % acrylamide gel with subsequent silver staining to confirm the presence of insert.

e) Plasmid transformation in DR1

Transformation of DR1 was performed by the calcium chloride method as described by Satoh et al., (2009). Briefly, DR1 cells (1 ml) grown to early stationary phase (16 h of approx. 1.2 $OD_{600\ nm}$) were harvested by centrifugation, at 9650 g or 3 min, washed with 1 ml of TGY broth, resuspended in 0.1 ml of TGY broth, amended with 40 µl of 0.3 M $CaCl_2$. A 30 µl aliquot of the cell mixture and 10 µl of plasmid DNA (200–400 µg per µl) were mixed in a new culture tube and incubated at 30°C for 90 min. To this was then added 2 ml of TGY broth and the mixture was incubated at 30 °C for 24 h. The culture was appropriately diluted with 10 mM sodium phosphate buffer (pH 7.0), spread on TGY plates supplemented with 3µg /ml chloramphenicol.The transformants were scored after 3-5 days of incubation at 30 °C.

4.2.7 Expression analysis of smt A in E.coli BL21 (DE3)

E. coli BL-21 (DE3) cells harbouring the expression plasmid, pMHNR1.1, were grown to an OD_{600} of approximately 0.4 to 0.5. Gene expression was induced by treatment with 1 mM IPTG (isopropyl-1-thio-L-D-galactopyranoside) and cells were further incubated for 3 h at 37 ^{0}C. The cells were centrifuged at 9600 g for 3 min to remove the supernatant and washed with Phosphate Buffer Saline (PBS; containing 137mM NaCl, 2.7mM KCl, 10mm Na_2HPO_4 and 2mM KH_2PO_4 pH 7.4). Cells were stored as frozen pellet at -20 ^{o}C. Cells were stored as frozen pellets at -20 ^{o}C. The thawed cells were disrupted by sonication in 10mM PBS, centrifuged at 12,000 g for 10 min. The supernatant was collected and mixed with 5x gel loading dye, boiled for 2-3 min and analysed on 15 % SDS-PAGE for the expression of recombinant protein with against un-induced cultures. Protein estimation was done using Bradford's protein estimation method (Bradford, 1976)

4.2.8 SDS-PAGE analysis

Proteins were fractionated and analysed on 15 % SDS-PAGE using standard procedures (Sambrook and Russell, 2001).

4.2.9 Cd^{2+} tolerance

Cd^{2+} tolerance of the isolates was examined by D_{50} determination as explained in Section 3.2.3.

4.2.10 Metal estimation by ICP-AES

50 ml cultures were grown in TGY to saturation in presence or absence of Cd^{2+}. The cultures were pelleted at 12,300 g for 5 min and washed twice with saline. The pellet was the dried at 60 °C overnight in pre-weighed glass vials. Pelleted cells were digested overnight in 1ml of digesting solution (1:1 mixture of 50 % HNO_3 (v/v) and 30% (w/v) H_2O_2) at 60 °C. The volume was increased to 10 ml with H_2O and filtered through Whattman 3. The metal content was analysed by ICP-AES (Helbig et al., 2008).

4.3 Results and Discussion

An attractive strategy is to develop metal resistance in organisms is to clone and express synthetic genes encoding protein analogs of PC with the general structure (Glu-Cys) $_n$ Gly (ECs). These peptides differ from PCs because the peptide bond between glutamic acid and cysteine is the standard a peptide bond that can be synthesized on the ribosomal machinery. Synthetic phytochelatin unlike the metallothionein are synthesised by the ribosomal machinery and contain α peptide bond instead of the γ peptide bond characteristic of the phytochelatin simplifying the expression of synthetic PCs in several host bacteria.

4.3.1 Synthesis, amplification and cloning of synthetic phytochelatin, ec20

The oligonucleotides employed for the construction of synthetic phytochelatin consisted of glutamate cysteine repeats, occurring twenty times hence the name *ec*20.The sequence of the oligonucleotide was kept the same as described by Bae et al., (2000). A PCR based approach was adopted for the synthesis of phytochelatin as described in Fig. 4.1. The first round of PCR amplification included annealing of the complementary region of EC1 and EC2 followed by overlap extension by *Taq.* DNA polymerase to generate synthetic *ec*20 gene which was further amplified by the terminal primers. Fig. 4.2 shows the amplified fragment of the *ec*20. *ec*20 was cloned in the pTZ57R/T and the clones were analysed by *EcoR*I and *Hind*III digestion (Fig. 4.3 A). PCR amplification of the random clone was also carried out (Fig. 4.3 B) to confirm the presence of the clones. Confirmed clone pTZ57RT 6ST (6ST) was employed for further sub-cloning (Fig. 4.4).

4.3.2 Sub-cloning of ec 20 in pRADZ3

For expression of *ec* 20 in DR1 pRADZ3 was chosen wherein the *lac* Z is under the effect of pgroEL (Meima and Lidstrom, 2000). Fig. 4.5 depicts the strategy of cloning of *ec*20 in pRADZ3 and the representative map of the clones in pRAD-*EC.ec*20 was cloned in *Spe* I and *Xba*I site of pRADZ3 and the clones were confirmed using *Xho*I / *Hind* III (Fig. 4.6) and *Spe*I / *Xba*I digestion (Fig. 4.7A). The short listed clones were subsequently confirmed by PCR amplification (Fig. 4.7B).

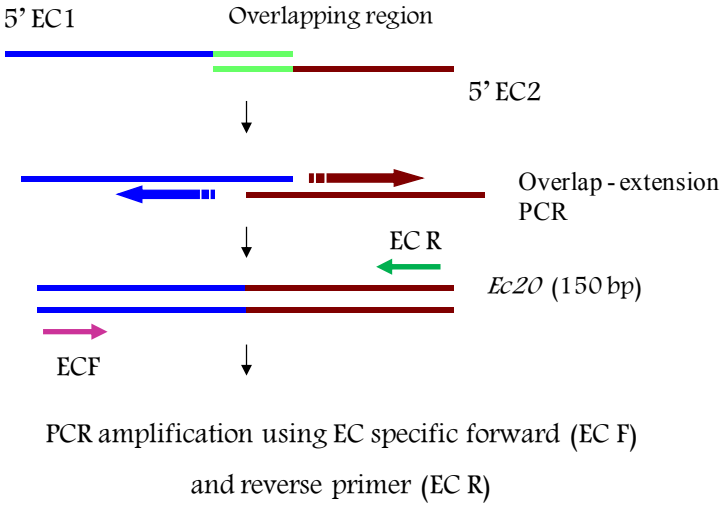


Fig. 4.1: Scheme for synthesis of *ec*20

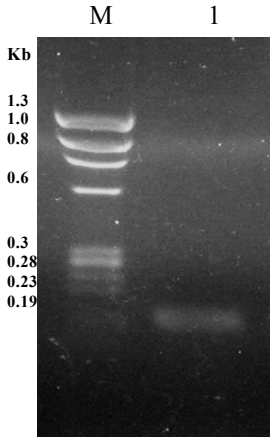


Fig. 4.2: Synthesis and PCR amplification of *ec*20.M: ϕX 174 DNA *Hae* III digest marker; 1: amplified *ec*20

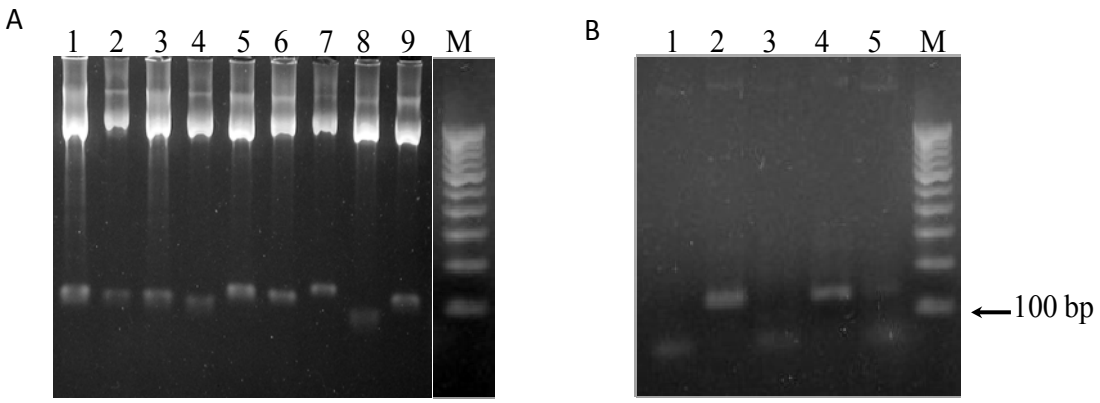


Fig. 4.3: T-vector clone confirmation of *ec*-20.A) Restriction digestion, *Eco*RI and *Xba*I; Lane 1-9: clones 1-9; M: 100 bp ladder; B) PCR amplification Lane 1: Negative control; Lane 2: Clone 6, 6ST; Lane 3: Clone 7,7ST; Lane 4: Clone 13, 13 ST; Lane 5: Clone 1, 1ST; Lane M: 100 bp marker.

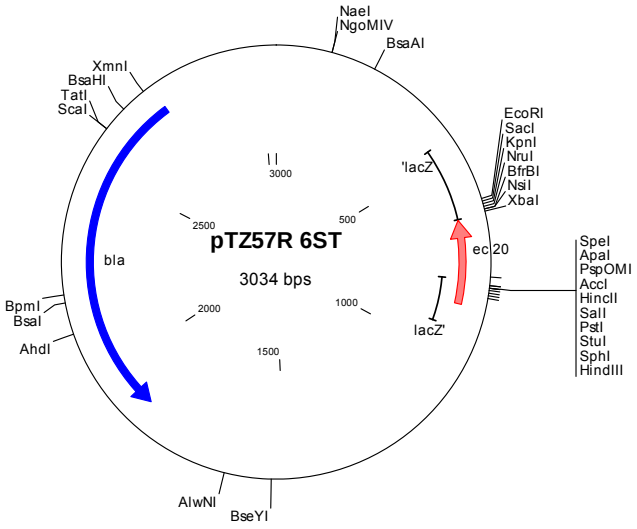


Fig. 4.4: Vector map of pTZ57R-6ST

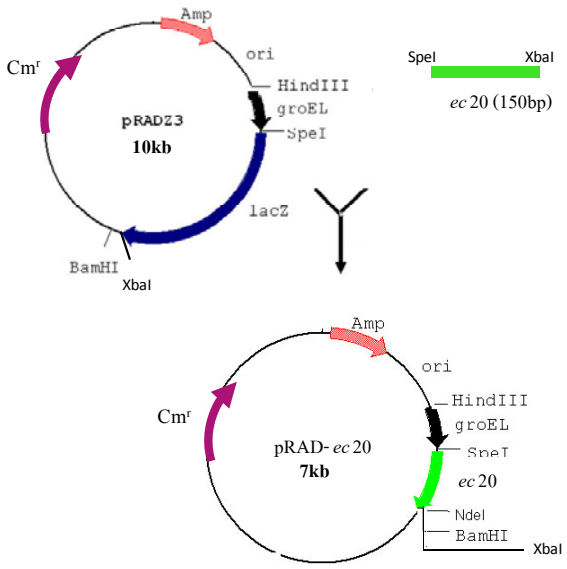


Fig. 4.5: Cloning strategy of *ec*20 in pRADZ3 and representative map of pRAD-EC clone

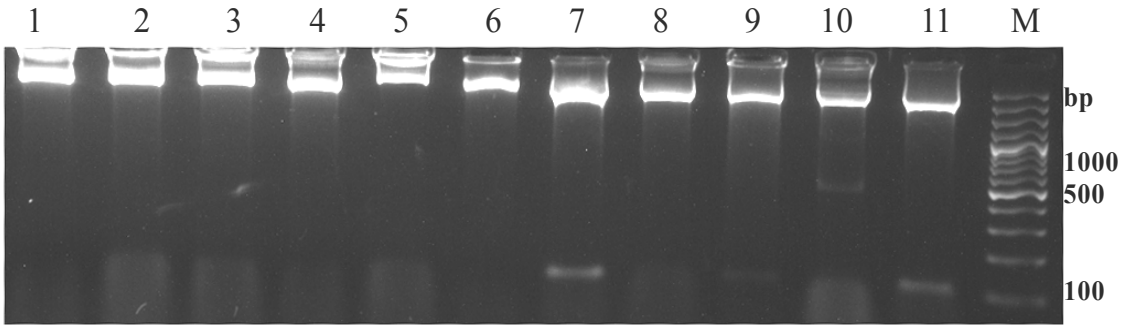


Fig. 4.6: Clone confirmation of *ec*20 obtained in pRADZ3 using *Xho*I and *Hind*III of representative clones.

Lane 1: Clone pRAD-1EC; Lane2: Clone pRAD-*3EC*; Lane3: Clone pRAD-*4EC*; Lane4: Clone pRAD-5*EC*; Lane5: Clone pRAD-6*EC*; Lane6: Clone pRAD-*7EC*; Lane7: Clone pRAD-9*EC*; Lane8: Clone pRAD-*10EC*; Lane9: Clone pRAD-*11EC*; Lane10: Clone pRAD-*12EC*; Lane11: Clone pRAD-*13EC*; M: 100bp marker

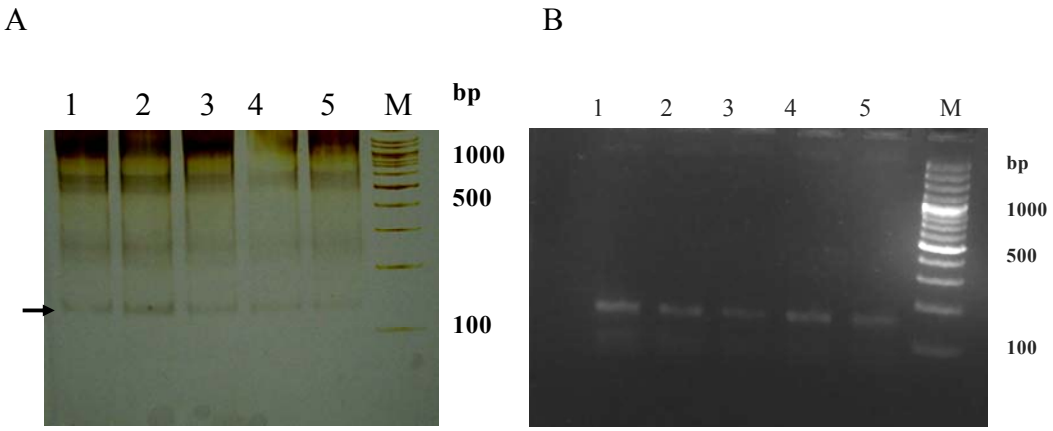


Fig. 4.7: Confirmation of ec 20 clones obtained in pRADZ3. A) using SpeI and XbaI; B) PCR amplification. Arrow indicates fragment corresponding to *ec* 20. Lanes in panel A and panel B are listed below. Lane1: Clone pRAD-*9EC*; Lane2: Clone pRAD-*11EC;* Lane3: Clone pRAD-*13EC*; Lane 4: Clone pRAD-*14EC*; Lane 5: Clone pRAD-*18EC*.

4.3.3 Construction of pRAD-smt A for heterologous expression in DR1

*smt*A is a novel MT and its coordination chemistry is of significance in view of the high affinity for Zn^{2+} and the intracellular exclusively handling of Zn^{2+} (Daniel et al., 1998).The efficacy of the *smt* A has been demonstrated for chelating Zn^{2+} and Cd^{2+}. Heterologous expression of *smt*A in DR1 was carried out to compare the efficiency of chelating Cd^{2+} by naturally occurring MT and synthetic MT. Fig. 4.8 shows the amplification of *smt* A from pMHNR1.1. The expected band of approximately 200 bp was obtained.

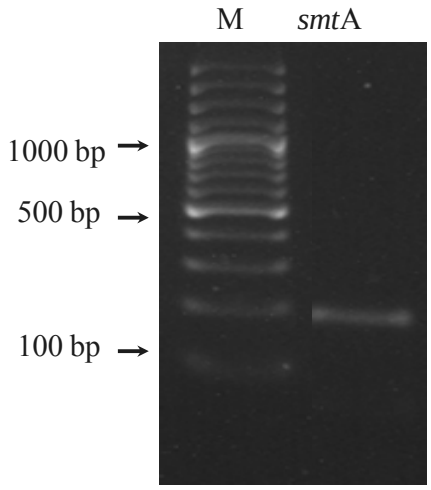


Fig. 4.8: PCR amplification of *smt* A from pMHNR1.1.M denotes 100 bp ladder.

pRADZ3 was digested with *Spe*I and *Bam*HI to relaease *lacZ* fragment of 3.2Kb. The linearised vector was purified and *smt* A was cloned in the *Spe*I, *Bam*HI site of pRADZ3. Fig. 4.9 depicts the strategy employed for cloning *smt*A in pRADZ3 and the vector map for the ensuing pRAD-*smt*A clones. The clones were randomly selected and confirmed using insert specific enzymes *Spe*I and *Bam*HI to confirm the orientation of the insert (Fig. 4.10) and subsequent confirmation was done using PCR amplification (Fig. 4.11).

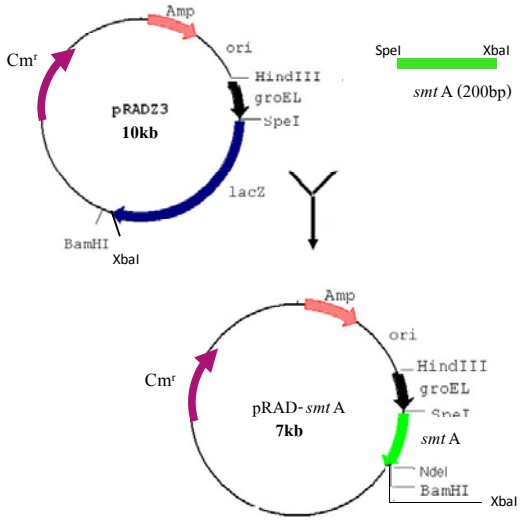


Fig. 4.9: Strategy employed for cloning smt A and representative map for the pRAD-*smt*A clones

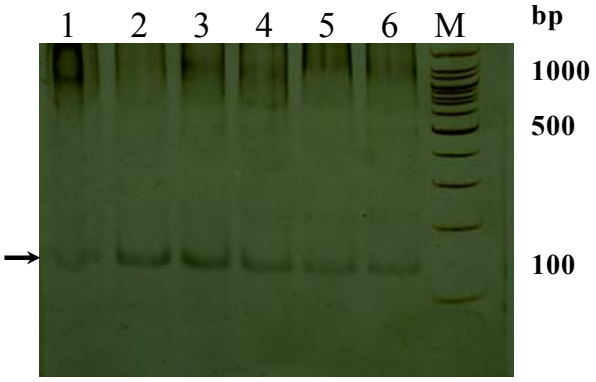


Fig. 4.10: Clone confirmation of pRAD-*smt*A using *Spe* I and *Bam*HI.Lane1: pRAD-*13 smtA*; Lane 2: pRAD-*18 smtA*; Lane 3: pRAD-*19 smtA*; Lane 4: pRAD-*22 smtA*; Lane5: pRAD-*23 smtA*; Lane6: pRAD-*24 smtA* ; M: 100bp ladder

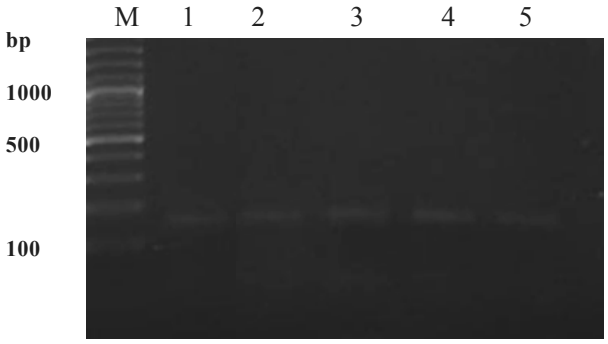


Fig. 4.11: Clone confirmation of pRAD-*smt*A by PCR Amplification.Lane1: pRAD-*13 smtA*; Lane 2: pRAD-*18 smtA*; Lane 3: pRAD-*19 smtA*; Lane 4: pRAD-*22 smtA*; Lane5: pRAD-*23 smtA*; M: 100 bp ladder

4.3.4 Transformation and expression analysis of pRAD-EC and prad-smtA in DR1

The plasmid DNA from confirmed clones was transformed in DR1 individually to generate DR1 (pRAD-*EC*) and DR1 (pRAD-*smt* A). SDS-PAGE analysis of the transformants showed an expected band of 4.5Kda for both DR1 (pRAD-*EC*) and DR1 (pRAD-*smt* A) (Fig. 4.12). *E. coli* BL-21 (DE3) carrying pMHNR1.1 was utilised as a positive control. Although the reported size of SmtA is 5.6 kDa (Blindauer et. al., 2001) a single induced band of > 4.5 kDa in the induced cultures of pMHNR1.1, not detectable in the control uninduced plasmid, was observed. From the translated sequence analysis of *ec*20, it has 32 aa which corresponds to expected molecular weight of 3.5 kDa. The observed molecular weight is in agreement with the expected molecular weight of the expressed protein (Fig. 4.12).

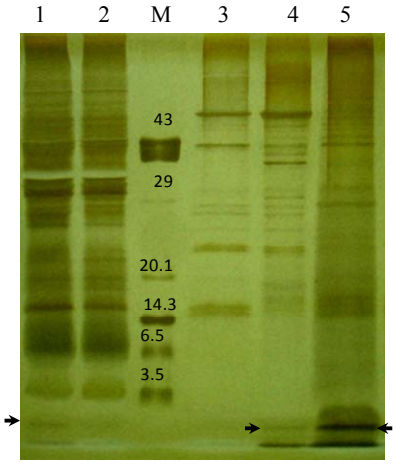


Fig. 4.12: Expression analysis of the DR1 transformants carrying MT. The numbers in the M lane indicate molecular weight in kDa. Lane1: *E.coli* (pMHNR1.1) induced; Lane2: *E.coli* pMHNR1.1 uninduced; Lane M: Low range molecular weight marker; Lane 3: DR1-pRADZ3

vector control; Lane 4: DR1-pRAD EC; Lane 5: DR1-pRAD smtA). The expressed bands are indicated by arrows. Molecular weights of each band in molecular weight marker are indicated above each band.

4.3.5 Characterization DR1 (pRAD-EC) and DR1 (pRAD-smtA)

In pRAD EC and pRAD *smt* A the respective genes are under the influence of the groESL promoter. Schimd and Lidstrom (2002) demonstrated that activity of the promoter groESL, as assayed by *lacZ* assay, was not significantly affected at 40 °C and the activity was similar to that determined by 30 °C. However, Holland et al., (2006) demonstrated that best expression from *groES* was obtained at 37 °C.Therefore all further experiments were carried out 37 °C. The growth kinetics was analysed for the vector control and transformants at 37 °C. The expression of *ec*20 and *smt*A only slightly enhanced the growth of DR1 at 37 °C (Fig. 4.13).

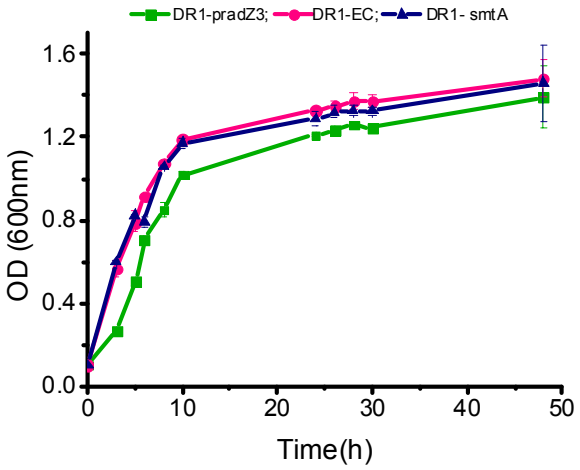


Fig. 4.13: Growth kinetics of DR1 (pRAD-EC) and DR1 (pRAD- *smt*A)

4.3.5aCd^{2+} tolerance ofDR1 (pRAD-EC) and DR1 (pRAD-smtA)

The most important functions of MTs in biological systems are their ability to chelate heavy metals. In most animal and plant cells they are particularly upregulated in presence of Cd^{2+}. The capacity of the transformants to tolerate the Cd^{2+} stress was analysed. At the concentrations analysed, the enhanced growth of DR1 (pRAD-EC) indicate the improved capacity to chelate Cd^{2+}. The natural MT, *smt*A clone was comparable to the synthetic MT. However at concentrations >10µM no significant effect on survival was observed (Fig. 4.14).

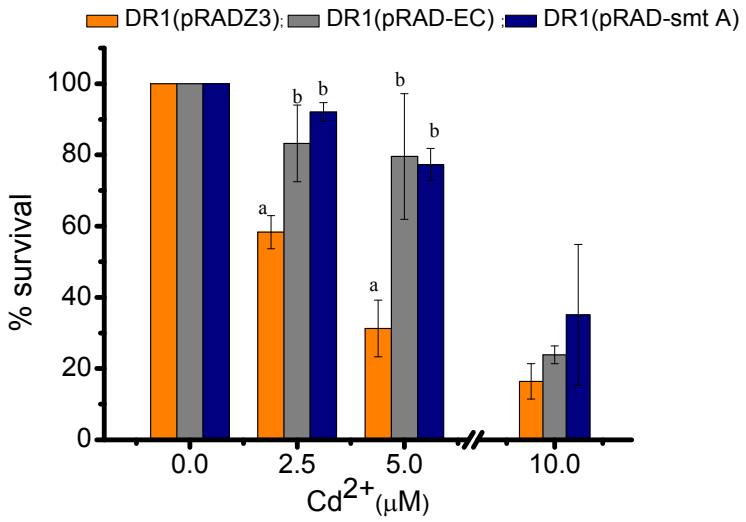


Fig. 4.14: Cd^{2+} tolerance of MT expressing transformants of DR1. Fisher-LSD was carried out to determine the significant difference in metal accumulation. Bars with similar alphabet do not differ significantly (LSD test p< 0.007).

4.3.5b Bioaccumulation of Cd^{2+} by DR1 (pRAD-EC) and DR1 (pRAD-smtA)

The expected outcome of the cloning *ec*20 and *smt* A was to sequester Cd^{2+} in DR1 and to improve its survivability in presence of Cd^{2+}. Hence the metal sequestration with respect to Cd^{2+} of the transformants was examined. Fig. 4.15 demonstrates Cd^{2+} accumulation by DR1 (pRAD-EC) and DR1 (pRAD-smtA). As compared to vector control, both MT expressing strain accumulated greater Cd^{2+}. *smt*A expressing strains, DR1 (pRAD-*smt*A), accumulated significantly higher amount of Cd^{2+} as opposed to the DR1 (pRAD-EC), expressing synthetic phytochelatin, *ec* 20, Intracellular levels of Cd^{2+} in DR1 (pRAD-smtA) were 300 % higher than the control while DR1 (pRAD-EC) accumulated 121 % higher than the control.

DR1 demonstrated an enhanced accumulation of Cd^{2+} when synthetic phytochelatin was expressed intracellularly however as opposed to the cytoplasmic expression of synthetic phytochelatin in *E.coli* (Pazirandeh et al., 1995)DR1 accumulated 6 fold lesser amount of Cd^{2+}. Most of the reports of expression of synthetic phytochelatin are either displayed on the surface or periplasmically in bacteria and are capable of enhanced chelation of the Cd^{2+} and cellular deposition of Cd^{2+} (Bae et al., 2002; Bae et al., 2000; Xu and Lee, 1999; Veils et al., 1998) (Table 4.2). Bae et al., (2002) reported enhanced accumulation of Cd^{2+} when the phytochelatin was expressed as fusion to ice nucleation protein of *Psuedomonas* spp. as opposed to periplasmic expression of the same.

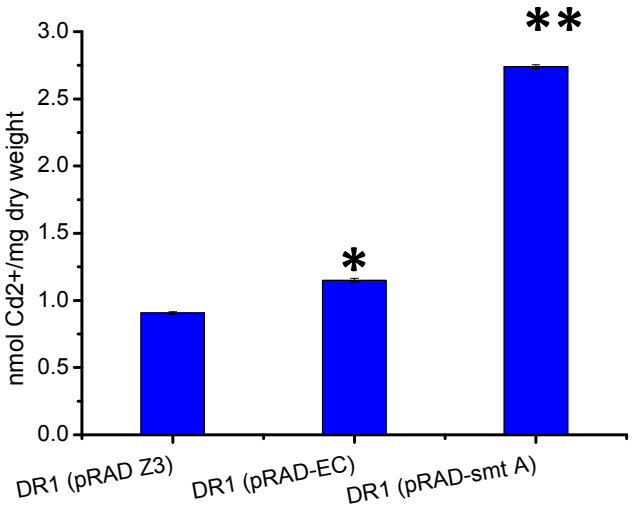


Fig. 4.15: Bioaccumulation of Cd^{2+} by MT transformants of DR1. Fisher-LSD was carried out determine the significant difference in metal accumulation P< 0.03 is indicated by single asterisk while P<0.001 is indicated by double asterisk.

The intracellular expressed metallothionein chelate less heavy metal as opposed to expression of the same MT in the periplasmic space which was attributed to enhanced oxidative stress produced that can oxidise the MT releasing the bound heavy metal exacerbating the ROS in the cell (Achard-Joris et al., 2007).

4.3.5c Effect of exogenous cysteine on the recombinant strain

*smt*A contains a smaller proportion of Cys than synthetic MT (16 % vs. 50 % of residues), and, unlike synthetic MTs, contains His residues, which have been implicated in metal coordination. (Blindauer et al., 2001). Lesser bioaccumulation in synthetic MT was observed which might be as result of the higher cysteine requirement of the cells for synthesising MT. As a result of the binding of Cd^{2+} to sulfide, generated during the biosynthesis of cysteine and of iron-sulfur centres (FeS centers); binding to thiol groups, e.g., of proteins; and the replacement of other transition-metal cations from such sulfur-rich complex compounds (Helbig et al., 2008), the requirement of cysteine may be aggravated in the cells expressing MT in presence of Cd^{2+}. It was hypothesized that the addition of cysteine would relieve the metabolic load and may restore the cells capacity to produce MT. Cysteine when added to a final concentration of 0.4 mM, showed an enhanced growth of the transformants as opposed to the vector control (Fig. 4.16). Addition of cysteine at higher concentrations resulted in toxicity to the vector control cells as compared to the transformants which were able to show growth up to 10 μM.

Table 4.2 Metal binding proteins and peptides and their effect on Cd^{2+} accumulation

Peptide/Protein	Expression site	Effect	Ref.
[a]His6, single or tandem expressed	OM, LamB	Five- and 11 fold increase in Cd^{2+} accumulation	Sousa et al., (1996)
[a]Human MT	OM, Lpp	66-fold increase in Cd^{2+}accumulation	Jacobs et al., (1989)
[a]Mammalian MT	OM, LamB	15–20 fold increase in Cd^{2+} accumulation	Sousa et al., (1998)
[a]CdBP (HSQKVF)	OM, OmpA	Increased Cd^{2+} tolerance	Mejáre et al., (1998)
[a]CP (GCGCPCGCG)	OM, LamB	Fourfold increase in Cd^{2+} accumulation	Pazirandeh et al., (1998)
[a]HP $(GHHPHG)_2$	OM, LamB	Threefold increase in Cd^{2+} accumulation	Kotrba, et al. (1999)
[a]MT α-domain	OM, LamB	17 fold increase in Cd^{2+} accumulation	Kotrba et al., (1999)
[a]1-12 tandem repeats of protein *Neurospora crassa* MT (CGCCG)	Periplasm, maltose binding	10–65 fold increase in Cd^{2+} accumulation	Mauro and Pazirandeh, (2000)
[a]EC(20), synthetic phytochelatin, (Glu-Cys)20Gly	OM, Lpp	30 fold increase in Cd^{2+} accumulation	Bae et al., (2000)
[b]MerR, MBD	Cytoplasmic	Increased tolerance to Hg^{2+}	Quin et al., (2006)
[b] EC(20), synthetic phytochelatin,(Glu-Cys)20	Cytoplasmic	1.21 fold increase in Cd^{2+} accumulation	This study
[b] Prokaryotic MT, Smt A	Cytoplasmic	3 fold increase in Cd^{2+} accumulation	This study

Abbreviations: OM, outer membrane; LamB, Calcium-binding; Lpp, protein; OmpA, CP, cysteine-containing peptide; HP, histidine-containing; MBD: Metal binding domain peptide.

[a]Expression in *E. coli*; [b] Expressed in DR1

. DR1 (pRAD-*smt*A) was able to tolerate greater Cd^{2+} as compared to the DR1(pRAD-EC) similar to that observed for TGY. When compared to growth in TGY, exogenous cysteine boosted the growth by 1.5 fold for vector control as well as for both the transformants. Cysteine amendment rescued Cd^{2+} toxicity for DR1 (pRAD-*smt*A) at all concentrations and the tolerance to Cd^{2+}at 10μM comparable to that of TGY. The growth of DR1 (pRAD-EC) and control was retarded in presence of

cysteine and Cd^{2+} (Fig. 4.17). Bioaccumulation of Cd^{2+} was determined for the transformants as well as the vector control. Fig. 4.18 illustrates the Cd^{2+} content of the MT expressing strains.

Exogenous cysteine didn't improve the Cd^{2+} sequestration, on the contrary the DR1 (pRAD-EC) accumulated least Cd^{2+}, accumulating only 65 % of the control while vector control and DR1 (pRAD-*smt* A) accumulated comparable amount of Cd^{2+}. The vector control accumulated less Cd^{2+} in presence of the cysteine as opposed to the same when grown in TGY only. A possible explanation of the reduced Cd^{2+} accumulation could be extracellular chelation of Cd^{2+} by cysteine (Singh and Pandey, 1981) and hence enhanced growth in presence of Cd^{2+}.Reduced accumulation of synthetic MT could not be explained.

Holland et al., (2006) demonstrated that methionine synthesis proceeds via the B_{12}-dependent enzyme, methionine synthase (MetH, DR0966), rather than the B_{12}-independent MetE and has an incomplete vitamin B12 pathway making the wild type DR1 an auxotroph for methionine. Therefore reduced intracellular Cd^{2+} by EC 20 expressing strains was attributed to the enhanced metabolic load due to increased cysteine demand in DR1. There is an expected competition for homocysteine pool for the conversion to methionine and cysteine during Cd^{2+}toxicity as indicated in Fig. 4.19.

4.4 Conclusion

This chapter deals with the expression of synthetic phytochelatin *ec*20 as well as natural prokaryotic metallothionein *smt* A in DR1. Qin et al., (2006) reported the cloning of metal binding domain of MerR and intercellular expression of the same in DR1 which allowed better survival of the transformants in presence of Hg^{2+}. The synthetic phytochelatin was constructed using overlap extension PCR and its subsequent expression in DR1 conferred the transformant, DR1 (pRAD-EC), 1.5 fold higher tolerance to Cd^{2+}. DR1 (pRAD-EC) accumulated 1.21 fold greater Cd^{2+} as opposed to the control. Heterologous expression of natural metallothionein, smtA, in DR1 imparted the transformants, DR1 (pRAD-*smt*A), superior tolerance to Cd^{2+} wherein DR1 (pRAD-*smt*A), amassed 2.5 fold greater Cd^{2+} than DR1-EC. Addition of cysteine enhanced the growth of the DR1 strains harbouring the metallothionein however it did not translate into efficient metal accumulation. The cysteine content of MTs is very high, which might interfere with cellular redox pathways in the cytosol (Raina and Missakis, 1997; Park and Imlay, 2003) may explain the observation.

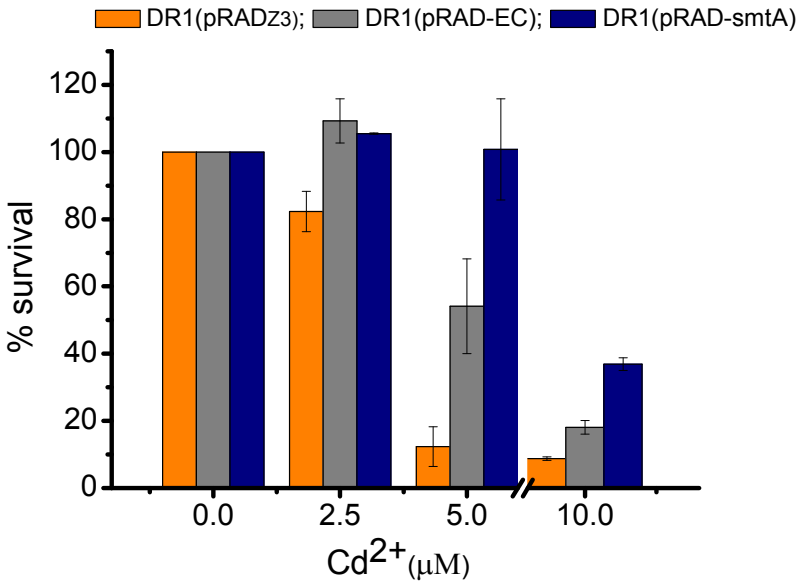


Fig. 4.16: Effect of cysteine on Cd2+ tolerance on MT expressing transformants of DR1.

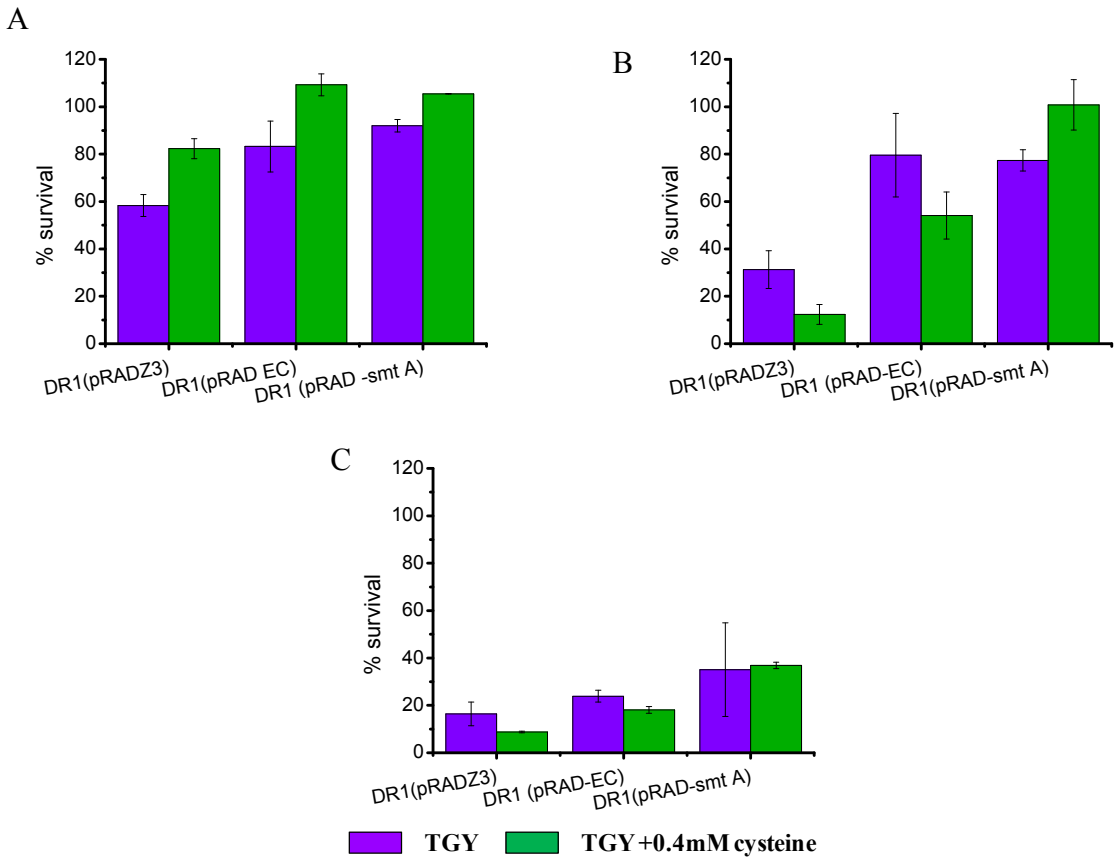


Fig. 4.17: Cd $^{2+}$ tolerance of DR1 expressing MT in presence and absence of 0.4 mM cysteineA) 2.5 µM Cd^{2+}; B) 5.0 µM Cd^{2+}; C) 10.0 µM Cd^{2+};

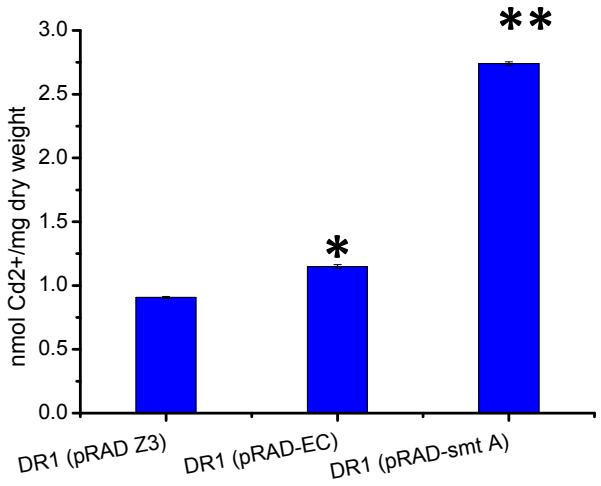


Fig. 4.18: Bioaccumulation of Cd^{2+} by MT transformants of DR1 in presence of cysteine. Fisher-LSD was carried out determine the significant difference in metal accumulation P< 0.007 is indicated.

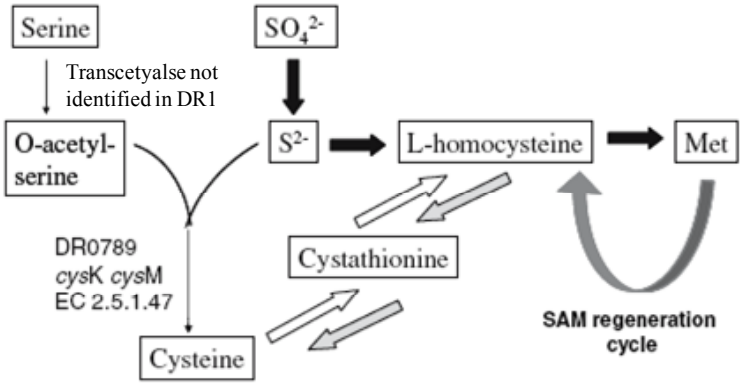


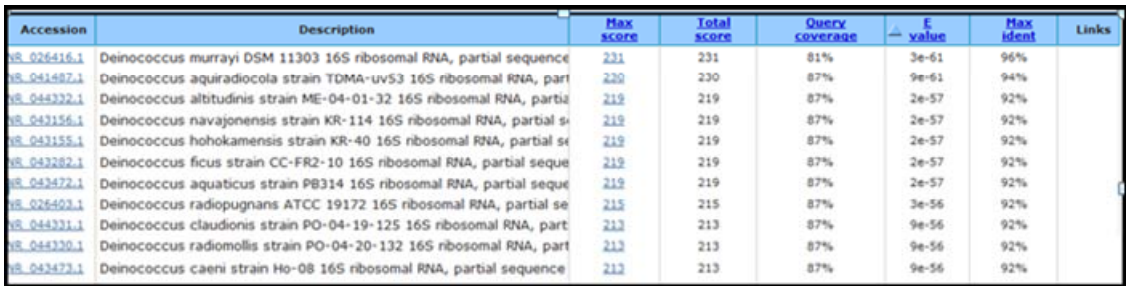
Fig. 4.19: Sulfur recycling in DR1.Black arrows indicates the pathway identified in DR1. (Holland et al., 2006)

Intracellular expression of MTs is, however, not devoid of complications and in many instances there have been problems with the stability and short half-life of the expressed heterologous proteins. Expressing MTs fused to a surface protein in DR1 can prove to be an attractive strategy for enhanced bioaccumulation of heavy metals including Hg^{2+}, Cu^{2+} and possibly other thiol-interacting metals.

Appendices

The more original a discovery, the more obvious it seems afterwards.
- Arthur Koestler

Appendix I: 16S rDNA sequence of the DGGE band from the environmental samples



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NR_043156.1	Deinococcus navajonensis strain KR-114 16S ribosomal RNA, partial s	219	219	87%	2e-57	92%	
NR_043155.1	Deinococcus hohokamensis strain KR-40 16S ribosomal RNA, partial se	219	219	87%	2e-57	92%	
NR_043282.1	Deinococcus ficus strain CC-FR2-10 16S ribosomal RNA, partial seque	219	219	87%	2e-57	92%	
NR_043472.1	Deinococcus aquaticus strain PB314 16S ribosomal RNA, partial seque	219	219	87%	2e-57	92%	
NR_026403.1	Deinococcus radiopugnans ATCC 19172 16S ribosomal RNA, partial se	215	215	87%	3e-56	92%	
NR_044321.1	Deinococcus claudionis strain PO-04-19-125 16S ribosomal RNA, part	213	213	87%	9e-56	92%	
NR_044320.1	Deinococcus radiomollis strain PO-04-20-132 16S ribosomal RNA, part	213	213	87%	9e-56	92%	
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AUTHORS     Chaturvedi,R., Das,S. and Archana,G.
TITLE       Development of a heminested polymerase chain reaction
protocol for the detection of Deioncoccus species from environmental
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JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 175)
AUTHORS     Archana,G. and Chaturvedi,R.
TITLE       Direct Submission
JOURNAL   Submitted (14-DEC-2008) Department of Microbiology and
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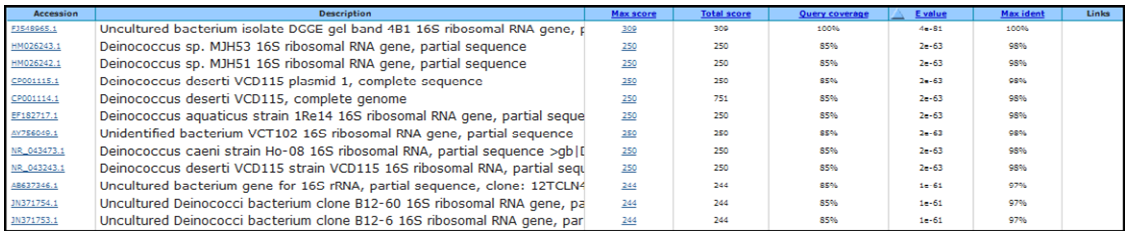

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TITLE         Development of a heminested polymerase chain reaction
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  TITLE     Direct Submission
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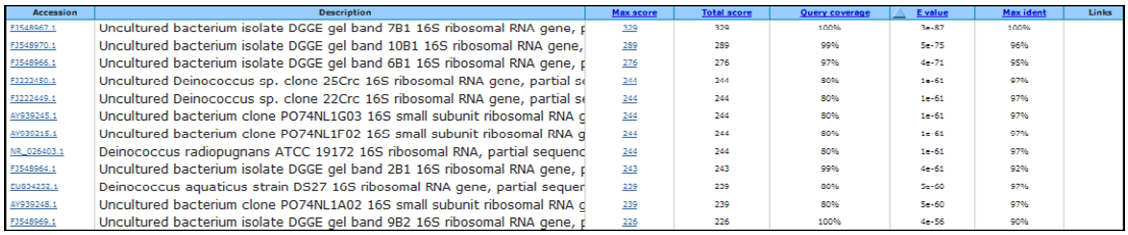

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  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 178)
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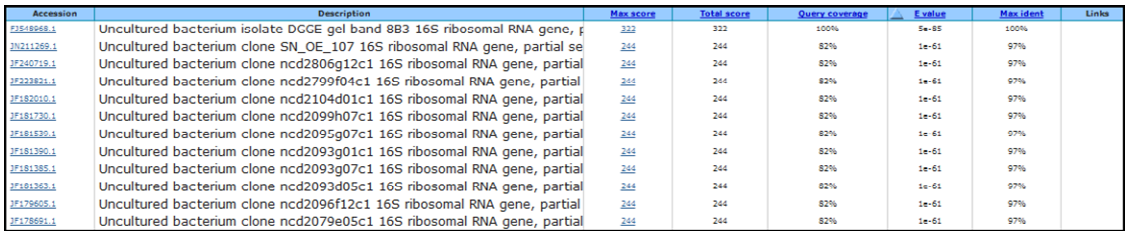

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AUTHORS     Chaturvedi,R., Das,S. and Archana,G.
TITLE         Development of a heminested polymerase chain reaction
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JOURNAL    Unpublished
REFERENCE   2  (bases 1 to 174)
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TITLE       Direct Submission
JOURNAL     Submitted (14-DEC-2008)
            Department of Microbiology and
            Biotechnology Center, The M.S. University of Baroda,
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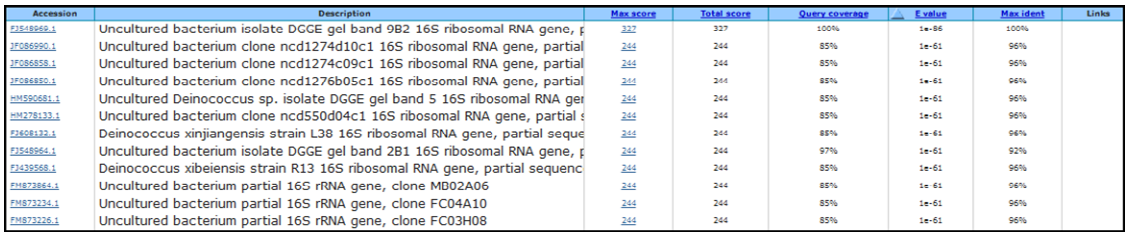

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HM278133.1	Uncultured bacterium clone ncd550d04c1 16S ribosomal RNA gene, partial s	244	244	85%	1e-61	96%	
FJ608132.1	Deinococcus xinjiangensis strain L38 16S ribosomal RNA gene, partial seque	244	244	85%	1e-61	96%	
FJ548964.1	Uncultured bacterium isolate DGGE gel band 2B1 16S ribosomal RNA gene, p	244	244	97%	1e-61	92%	
FJ439568.1	Deinococcus xibeiensis strain R13 16S ribosomal RNA gene, partial sequenc	244	244	85%	1e-61	96%	
FM873864.1	Uncultured bacterium partial 16S rRNA gene, clone MB02A06	244	244	85%	1e-61	96%	
FM873234.1	Uncultured bacterium partial 16S rRNA gene, clone FC04A10	244	244	85%	1e-61	96%	
FM873226.1	Uncultured bacterium partial 16S rRNA gene, clone FC03H08	244	244	85%	1e-61	96%	

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LOCUS       FJ548969          177 bp    DNA     linear   ENV 31-JAN-
2009
DEFINITION   Uncultured bacterium isolate DGGE gel band 9B2 16S
ribosomal RNA
gene, partial sequence.
ACCESSION   FJ548969
VERSION     FJ548969.1  GI:221326901
KEYWORDS    ENV.
SOURCE      uncultured bacterium
ORGANISM  uncultured bacterium
Bacteria; environmental samples.
REFERENCE   1  (bases 1 to 177)
AUTHORS     Chaturvedi,R., Das,S. and Archana,G.
TITLE         Development of a heminested polymerase chain reaction
            protocol for the detection of Deioncoccus species from
            environmental samples
  JOURNAL   Unpublished
REFERENCE   2 (bases 1 to 177)
  AUTHORS   Archana,G. and Chaturvedi,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-DEC-2008) Department of Microbiology and
            Biotechnology Center, The M.S. University of Baroda,
Sayajigunj,
            Vadodara, Gujarat 3900 02, India
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                     /product="16S ribosomal RNA"
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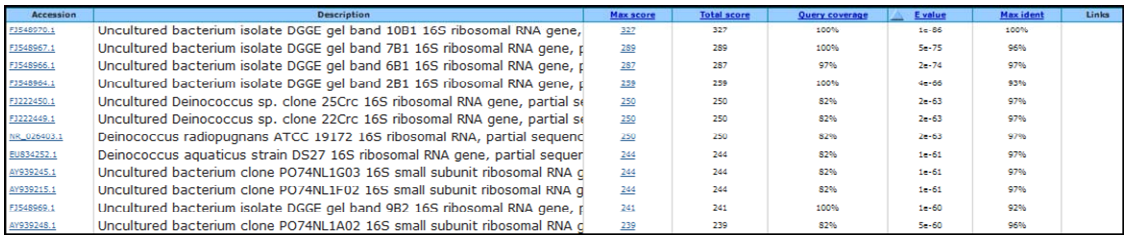

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FJ548967.1	Uncultured bacterium isolate DGGE gel band 7B1 16S ribosomal RNA gene, p	289	289	100%	5e-75	96%	
FJ548966.1	Uncultured bacterium isolate DGGE gel band 6B1 16S ribosomal RNA gene, p	287	287	97%	2e-74	97%	
FJ548964.1	Uncultured bacterium isolate DGGE gel band 2B1 16S ribosomal RNA gene, p	259	259	100%	4e-66	93%	
FJ222450.1	Uncultured Deinococcus sp. clone 25Crc 16S ribosomal RNA gene, partial s	250	250	82%	2e-63	97%	
FJ222449.1	Uncultured Deinococcus sp. clone 22Crc 16S ribosomal RNA gene, partial s	250	250	82%	2e-63	97%	
NR_026403.1	Deinococcus radiopugnans ATCC 19172 16S ribosomal RNA, partial sequenc	250	250	82%	2e-63	97%	
EU834252.1	Deinococcus aquaticus strain DS27 16S ribosomal RNA gene, partial sequer	244	244	82%	1e-61	97%	
AY939245.1	Uncultured bacterium clone PO74NL1G03 16S small subunit ribosomal RNA g	244	244	82%	1e-61	97%	
AY939215.1	Uncultured bacterium clone PO74NL1F02 16S small subunit ribosomal RNA g	244	244	82%	1e-61	97%	
FJ548969.1	Uncultured bacterium isolate DGGE gel band 9B2 16S ribosomal RNA gene, p	241	241	100%	1e-60	92%	
AY939248.1	Uncultured bacterium clone PO74NL1A02 16S small subunit ribosomal RNA g	239	239	82%	5e-60	96%	

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LOCUS       FJ548970                177 bp    DNA     linear   ENV
31-JAN-2009
DEFINITION  Uncultured bacterium isolate DGGE gel band 10B1 16S
            ribosomal RNA gene, partial sequence.
ACCESSION   FJ548970
VERSION     FJ548970.1  GI:221326902
KEYWORDS    ENV.
SOURCE      uncultured bacterium
ORGANISM  uncultured bacterium
Bacteria; environmental samples.
REFERENCE   1  (bases 1 to 177)
  AUTHORS   Chaturvedi,R., Das,S. and Archana,G.
  TITLE     Development of a heminested polymerase chain reaction
protocol for
the detection of Deioncoccus species from environmental samples
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 177)
  AUTHORS   Archana,G. and Chaturvedi,R.
  TITLE     Direct Submission
  JOURNAL   Submitted (14-DEC-2008) Department of Microbiology and
            Biotechnology Center, The M.S. University of Baroda,
Sayajigunj,
            Vadodara, Gujarat 3900 02, India
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Appendix II: 16S rDNA from group specific library from the environmental samples.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
FJ189470.1	Uncultured Deinococcus sp. clone 6Crc 16S ribosomal RNA gene, partial se	1129	1129	100%	0.0	100%	
FJ222448.1	Uncultured Deinococcus sp. clone 19Crc 16S ribosomal RNA gene, partial s	998	998	99%	0.0	97%	
EU834252.1	Deinococcus aquaticus strain DS27 16S ribosomal RNA gene, partial sequer	985	985	97%	0.0	97%	
NR_074603.1	Deinococcus radiopugnans ATCC 19172 16S ribosomal RNA, partial sequenc	983	983	97%	0.0	97%	
AY571843.1	Deinococcus sp. 34/20 16S ribosomal RNA gene, partial sequence	950	950	97%	0.0	96%	
EU622978.1	Deinococcus sp. 5516T-9 16S ribosomal RNA gene, partial sequence	937	937	96%	0.0	95%	
EU308576.1	Deinococcus sp. 1A6 16s ribosomal RNA gene, partial sequence	937	937	96%	0.0	95%	
EU029126.1	Deinococcus sp. 1B3 16S ribosomal RNA gene, partial sequence	937	937	96%	0.0	95%	
DQ532164.1	Uncultured bacterium clone JPL2-78 16S ribosomal RNA gene, partial seque	937	937	96%	0.0	95%	
GQ339894.1	Deinococcus sp. BE4-4 16S ribosomal RNA gene, partial sequence >gb|GQ3	933	933	96%	0.0	96%	
JF124401.1	Uncultured bacterium clone ncd1436e01c1 16S ribosomal RNA gene, partial	931	931	96%	0.0	95%	
JF124332.1	Uncultured bacterium clone ncd1435e09c1 16S ribosomal RNA gene, partial	931	931	96%	0.0	95%	

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LOCUS        FJ189470         611 bp     DNA      linear    ENV 30-SEP-
2008
DEFINITION  Uncultured Deinococcus sp. clone 6Crc 16S ribosomal RNA
gene,
partial sequence.
ACCESSION   FJ189470
VERSION     FJ189470.1  GI:206601414
KEYWORDS    ENV.
SOURCE      uncultured Deinococcus sp.
ORGANISM  uncultured Deinococcus sp.
            Bacteria; Deinococcus-Thermus; Deinococci; Deinococcales;
Deinococcaceae; Deinococcus; environmental samples.
REFERENCE   1  (bases 1 to 611)
AUTHORS    Chaturvedi,R. and Archana,G.
TITLE        Development of a heminested polymerase chain reaction
            protocol for the detection of Deioncoccus species from
            environmental samples
JOURNAL    Unpublished
REFERENCE   2  (bases 1 to 611)
AUTHORS    Chaturvedi,R. and Archana,G.
TITLE      Direct Submission
JOURNAL    Submitted (06-SEP-2008) Department of Microbiology and
           Biotechnology Centre, The M.S. University of Baroda,
            Sayajigunj, Vadodara, Gujarat 390 002, India
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                     /country="India: Vadodara"
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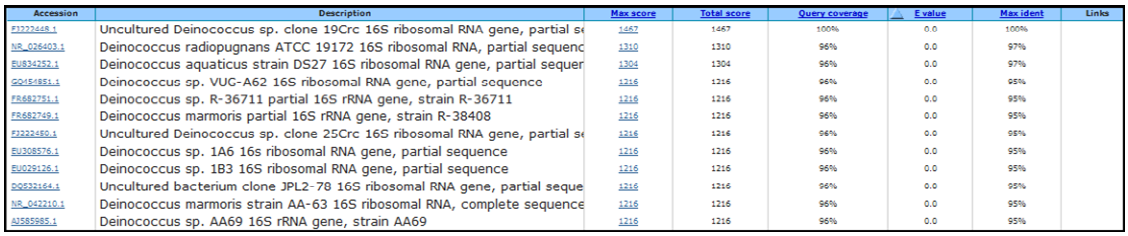

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FJ222448.1	Uncultured Deinococcus sp. clone 19Crc 16S ribosomal RNA gene, partial s	1667	1467	100%	0.0	100%	
NR_026403.1	Deinococcus radiopugnans ATCC 19172 16S ribosomal RNA, partial sequenc	1310	1310	96%	0.0	97%	
EU834252.1	Deinococcus aquaticus strain DS27 16S ribosomal RNA gene, partial sequer	1304	1304	96%	0.0	97%	
GQ454851.1	Deinococcus sp. VUC-A62 16S ribosomal RNA gene, partial sequence	1216	1216	96%	0.0	95%	
FR682751.1	Deinococcus sp. R-36711 partial 16S rRNA gene, strain R-36711	1216	1216	96%	0.0	95%	
FR682749.1	Deinococcus marmoris partial 16S rRNA gene, strain R-38408	1216	1216	96%	0.0	95%	
FJ222450.1	Uncultured Deinococcus sp. clone 25Crc 16S ribosomal RNA gene, partial s	1216	1216	96%	0.0	95%	
EU308576.1	Deinococcus sp. 1A6 16s ribosomal RNA gene, partial sequence	1216	1216	96%	0.0	95%	
EU029126.1	Deinococcus sp. 1B3 16S ribosomal RNA gene, partial sequence	1216	1216	96%	0.0	95%	
DQ822164.1	Uncultured bacterium clone JPL2-78 16S ribosomal RNA gene, partial seque	1216	1216	96%	0.0	95%	
NR_042210.1	Deinococcus marmoris strain AA-63 16S ribosomal RNA, complete sequence	1216	1216	96%	0.0	95%	
AJ585985.1	Deinococcus sp. AA69 16S rRNA gene, strain AA69	1216	1216	96%	0.0	95%	

LOCUS FJ222448 794 bp DNA linear ENV 05-OCT-2008
DEFINITION Uncultured Deinococcus sp. clone 19Crc 16S ribosomal RNA gene, partial sequence.
ACCESSION FJ222448
VERSION FJ222448.1 GI:207298848
KEYWORDS ENV.
SOURCE uncultured Deinococcus sp.
ORGANISM uncultured Deinococcus sp.
Bacteria; Deinococcus-Thermus; Deinococci; Deinococcales; Deinococcaceae; Deinococcus; environmental samples.
REFERENCE 1 (bases 1 to 794)
AUTHORS Chaturvedi,R. and Archana,G.
TITLE Development of a heminested polymerase chain reaction protocol for the detection of Deioncoccus species from environmental samples
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 794)
AUTHORS Chaturvedi,R. and Archana,G.
TITLE Direct Submission
JOURNAL Submitted (10-SEP-2008) Department of Microbiology and Biotechnology Center, The M.S. University of Baroda, Sayajigunj,Vadodara, Gujarat 390 002, India
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/product="16S ribosomal RNA"
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121 gagggatgaa ggtttcggat cgtaaacctc tgaatctggg acgaaagacc gcgaagagcg
181 gagatgacgg taccagagta atagcaccgg ctaactccgt gccagcagcc gcggtaatac
241 ggagggtgca agcgttaccc ggaatcactg ggcgtaaagg gcgtgtaggc ggcctgccaa
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421 caccgatggc gaaggcaggt tcttggacag aaggtgacgc tgaggcgcga aagtgtgggg
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541 caggatgctg ttgtgggcga agctaacgcg ataaacgtac cgcctgggaa gtacggccgc
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661 ttcgaagcaa cgcgaagaac cttaccaggt cttgacatcc catgaacccc tgagagatca
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Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
FJ222449.1	Uncultured Deinococcus sp. clone 22Crc 16S ribosomal RNA gene, partial s	1452	1452	100%	0.0	100%	
NR_026403.1	Deinococcus radiopugnans ATCC 19172 16S ribosomal RNA, partial sequenc	1306	1306	99%	0.0	97%	
EU834252.1	Deinococcus aquaticus strain DS27 16S ribosomal RNA gene, partial sequer	1303	1303	99%	0.0	96%	
FJ222450.1	Uncultured Deinococcus sp. clone 25Crc 16S ribosomal RNA gene, partial s	1295	1295	99%	0.0	96%	
NR_042209.1	Deinococcus saxicola strain AA-1444 16S ribosomal RNA, complete sequenc	1245	1245	99%	0.0	95%	
FR682749.1	Deinococcus marmoris partial 16S rRNA gene, strain R-38408	1234	1234	99%	0.0	95%	
EU622978.1	Deinococcus sp. 5516T-9 16S ribosomal RNA gene, partial sequence	1234	1234	99%	0.0	95%	
NR_042210.1	Deinococcus marmoris strain AA-63 16S ribosomal RNA, complete sequence	1234	1234	99%	0.0	95%	
AJ585985.1	Deinococcus sp. AA69 16S rRNA gene, strain AA69	1234	1234	99%	0.0	95%	
EF093134.1	Deinococcus sp. VTT E-052909 16S ribosomal RNA gene, complete sequen	1229	1229	99%	0.0	95%	
AJ585983.1	Deinococcus sp. AA752 16S rRNA gene, strain AA752	1229	1229	99%	0.0	95%	
AJ585982.1	Deinococcus sp. AA829 16S rRNA gene, strain AA829	1229	1229	99%	0.0	95%	

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ACCESSION   FJ222449
VERSION     FJ222449.1  GI:207298849
KEYWORDS    ENV.
SOURCE      uncultured Deinococcus sp.
ORGANISM  uncultured Deinococcus sp.
            Bacteria; Deinococcus-Thermus; Deinococci; Deinococcales;
Deinococcaceae; Deinococcus; environmental samples.
REFERENCE   1  (bases 1 to 786)
AUTHORS   Chaturvedi,R. and Archana,G.
TITLE       Development of a heminested polymerase chain reaction
            protocol for the detection of Deioncoccus species from
            environmental samples
JOURNAL    Unpublished
REFERENCE   2  (bases 1 to 786)
AUTHORS    Chaturvedi,R. and Archana,G.
TITLE      Direct Submission
JOURNAL    Submitted (10-SEP-2008) Department of Microbiology and
           Biotechnology Center, The M.S. University of Baroda,
            Sayajigunj,Vadodara, Gujarat 390 002, India
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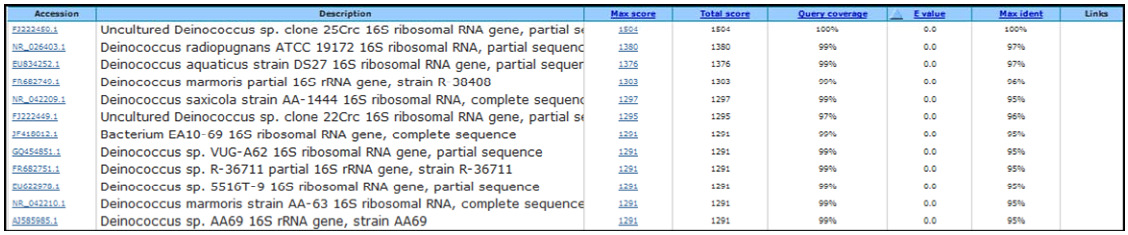

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
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NR_026403.1	Deinococcus radiopugnans ATCC 19172 16S ribosomal RNA, partial sequenc	1380	1380	99%	0.0	97%	
EU834252.1	Deinococcus aquaticus strain DS27 16S ribosomal RNA gene, partial sequer	1376	1376	99%	0.0	97%	
FN682740.1	Deinococcus marmoris partial 16S rRNA gene, strain R 30408	1303	1303	99%	0.0	96%	
NR_042209.1	Deinococcus saxicola strain AA-1444 16S ribosomal RNA, complete sequenc	1297	1297	99%	0.0	95%	
FJ222449.1	Uncultured Deinococcus sp. clone 22Crc 16S ribosomal RNA gene, partial s	1295	1295	97%	0.0	96%	
JF418612.1	Bacterium EA10-69 16S ribosomal RNA gene, complete sequence	1291	1291	99%	0.0	95%	
GQ456851.1	Deinococcus sp. VUG-A62 16S ribosomal RNA gene, partial sequence	1291	1291	99%	0.0	95%	
FR682751.1	Deinococcus sp. R-36711 partial 16S rRNA gene, strain R-36711	1291	1291	99%	0.0	95%	
EU622978.1	Deinococcus sp. 5516T-9 16S ribosomal RNA gene, partial sequence	1291	1291	99%	0.0	95%	
NR_042210.1	Deinococcus marmoris strain AA-63 16S ribosomal RNA, complete sequence	1291	1291	99%	0.0	95%	
AJ585985.1	Deinococcus sp. AA69 16S rRNA gene, strain AA69	1291	1291	99%	0.0	95%	

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LOCUS       FJ222450      814 bp    DNA     linear   ENV 05-OCT-2008
DEFINITION  Uncultured Deinococcus sp. clone 25Crc 16S ribosomal RNA
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ACCESSION   FJ222450
VERSION     FJ222450.1  GI:207298850
KEYWORDS    ENV.
SOURCE      uncultured Deinococcus sp.
ORGANISM    uncultured Deinococcus sp.
            Bacteria; Deinococcus-Thermus; Deinococci; Deinococcales;
Deinococcaceae; Deinococcus; environmental samples.
REFERENCE   1  (bases 1 to 814)
  AUTHORS   Chaturvedi,R. and Archana,G.
  TITLE      Development of a heminested polymerase chain reaction
            protocol for the detection of Deioncoccus species from
            environmental samples
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 814)
  AUTHORS   Chaturvedi,R. and Archana,G.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-SEP-2008) Department of Microbiology and
            Biotechnology Center, The M.S. University of Baroda,
            Sayajigunj,Vadodara, Gujarat 390 002, India
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301 cactgggcgt aaagggcgtg taggcggcct gccaagtctg gttttaaagc ctgcggctcg
361 accgcagaga tggactggag actggtaggc tagacctctg gagagagaac tggaattcct
421 ggtgtagcgg tggaatgcgt agataccagg aggaacaccg atggcgaagg caggttcttg
481 gacagaaggt gacgctgagg cgcgaaagtg tggggagcaa accggattaa tacccgggta
541 gtccacaccc taaacgatgt acgttggcct acagcaggat gctgttgtgg gcgaagctaa
601 cgcgataaac gtaccgcctg ggaagtacgg ccgcaaggtt gaaactcaaa ggaattgacg
661 ggggcccgca caagcggtgg agcatggggt ttaattcgaa gcaacgcgaa aaccttacag
721 gtcttgacat cccatgaacc ctgaaaaaca aggggtgccc ttcggggagc atgaacaggt
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Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
NR_043157.1	Deinococcus hopiensis KR-140 strain KR-140 16S ribosomal RNA, part	917	917	99%	0.0	91%	
NR_026401.1	Deinococcus radiodurans strain DSM 20539 16S ribosomal RNA, partia	894	894	99%	0.0	91%	
NR_044542.1	Deinococcus xinjiangensis strain X-82 16S ribosomal RNA, partial sequ	883	883	99%	0.0	91%	
NR_043743.1	Deinococcus yunweiensis strain YIM 007 16S ribosomal RNA, partial s	872	872	99%	0.0	90%	
NR_043282.1	Deinococcus ficus strain CC-FR2-10 16S ribosomal RNA, partial seque	856	856	99%	0.0	90%	
NR_029004.1	Deinococcus indicus strain Wt/1a 16S ribosomal RNA, partial sequenc	856	856	99%	0.0	90%	
NR_044497.1	Deinococcus gobiensis I-0 strain I-0 16S ribosomal RNA, partial seque	850	850	99%	0.0	90%	
NR_042696.1	Deinococcus aquatilis strain : CCUG 53370 16S ribosomal RNA, partial	850	850	99%	0.0	90%	
NR_043162.1	Deinococcus sonorensis KR-87 strain KR-87 16S ribosomal RNA, partia	850	850	99%	0.0	90%	
NR_043155.1	Deinococcus hohokamensis strain KR-40 16S ribosomal RNA, partial se	850	850	99%	0.0	90%	
NR_043243.1	Deinococcus deserti VCD115 strain VCD115 16S ribosomal RNA, partia	850	850	99%	0.0	90%	
NR_026399.1	Deinococcus grandis strain DSM 3963 16S ribosomal RNA, partial sequ	850	850	99%	0.0	90%	

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LOCUS       HQ738631                677 bp    DNA     linear   ENV
09-MAR-2011
DEFINITION  Uncultured Deinococcus sp. clone G1 16S ribosomal RNA
            gene, partial sequence.
ACCESSION   HQ738631
VERSION     HQ738631.1  GI:325296106
KEYWORDS    ENV.
SOURCE      uncultured Deinococcus sp.
ORGANISM    uncultured Deinococcus sp.
            Bacteria; Deinococcus-Thermus; Deinococci; Deinococcales;
Deinococcaceae; Deinococcus; environmental samples.
REFERENCE   1  (bases 1 to 677)
AUTHORS     Chaturvedi,R. and Archana,G.
TITLE       Development of a heminested polymerase chain reaction
            protocol for the detection of Deinococcus species from
            environmental samples
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 677)
  AUTHORS   Chaturvedi,R. and Archana,G.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-DEC-2010) Department of Microbiology and
            Biotechnology Centre, M.S. University of Baroda,
Sayajigunj,
            Vadodara, Gujarat 390002, India
FEATURES             Location/Qualifiers
source          1..677
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                     /db_xref="taxon:158789"
                     /clone="G1"
                     /environmental_sample
                     /country="India"
                     /note="PCR_primers=fwd_name: DeinoF, rev_name:
Eubacterial
                     1107R"
rRNA<1..>677
                     /product="16S ribosomal RNA"
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      121 ccgcggtaat acggagggtg caagcgttac ccggaatcac tgggcgtaca gggcgtgtag
      181 gcggacactt aagtctggtt ttaaagaccg gggctcaacc ccggaagagg actggatact
      241 gggtgtcttg acctctggag aggcaactgg aattcctggt gtagcggtgg aatgcgtaga
      301 taccaggagg aacaccaatg gcgaaggcag gttgctggac agaaggtgac gctgaggcgc
      361 gaaagtgtgg ggagcgaacc ggattagata cccgggtagt ccacacccta aacgatgtac
      421 gttggctcat ggcaggatgc tgtcatgggc gaagctaacg cgataaacgt accgcctggg
      481 aagtacggcc gcaaggttga aactcaaagg aattgacggg ggcccgcaca agcggtggag
      541 catgtggttt aattcgatga tacgcgagga accttaccta ggctagaatg cgcgtgaccg
      601 gagcagagat gctccttccc ttcggggcac aaagcaaggt gcggcatggc tgtcgccagc
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      661 tcgtgccgtg agggtgc
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Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
NR_026416.1	Deinococcus murrayi DSM 11303 16S ribosomal RNA, partial sequence	1378	1378	99%	0.0	96%	
NR_040934.1	Deinococcus aerius strain TR0125 16S ribosomal RNA, partial sequenc	1267	1267	99%	0.0	94%	
NR_043158.1	Deinococcus apachensis strain KR-36 16S ribosomal RNA, partial sequ	1256	1256	99%	0.0	94%	
NR_043472.1	Deinococcus aquaticus strain PB314 16S ribosomal RNA, partial seque	1245	1245	99%	0.0	93%	
NR_043157.1	Deinococcus hopiensis KR-140 strain KR-140 16S ribosomal RNA, part	1234	1234	99%	0.0	93%	
NR_041487.1	Deinococcus aquiradiocola strain TDMA-uv53 16S ribosomal RNA, part	1230	1230	98%	0.0	93%	
NR_042209.1	Deinococcus saxicola strain AA-1444 16S ribosomal RNA, complete se	1230	1230	99%	0.0	93%	
NR_043743.1	Deinococcus yunweiensis strain YIM 007 16S ribosomal RNA, partial s	1229	1229	99%	0.0	93%	
NR_043473.1	Deinococcus caeni strain Ho-08 16S ribosomal RNA, partial sequence	1223	1223	99%	0.0	93%	
NR_044542.1	Deinococcus xinjiangensis strain X-82 16S ribosomal RNA, partial sequ	1212	1212	99%	0.0	93%	
NR_042210.1	Deinococcus marmoris strain AA-63 16S ribosomal RNA, complete seq	1208	1208	99%	0.0	93%	
NR_042208.1	Deinococcus frigens strain AA-692 16S ribosomal RNA, complete sequ	1208	1208	99%	0.0	93%	

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LOCUS       HQ738632                 839 bp    DNA     linear   ENV
09-MAR-2011
DEFINITION  Uncultured Deinococcus sp. clone G4 16S ribosomal RNA
            gene,    partial  sequence.
ACCESSION   HQ738632
VERSION     HQ738632.1  GI:325296107
KEYWORDS    ENV.
SOURCE      uncultured Deinococcus sp.
ORGANISM  uncultured Deinococcus sp.
            Bacteria; Deinococcus-Thermus; Deinococci; Deinococcales;
Deinococcaceae; Deinococcus; environmental samples.
REFERENCE   1  (bases 1 to 839)
AUTHORS     Chaturvedi,R. and Archana,G.
TITLE        Development of a heminested polymerase chain reaction
            protocol      for the detection of Deinococcus species
            from environmental samples
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 839)
  AUTHORS   Chaturvedi,R. and Archana,G.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-DEC-2010) Department of Microbiology and
            Biotechnology   Centre,   M.S.   University   of   Baroda,
Sayajigunj,
            Vadodara, Gujarat 390002, India
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                     /isolation_source="Greater Rann of Kutch"
                     /db_xref="taxon:158789"
                     /clone="G4"
                     /environmental_sample
                     /country="India"
                     /note="PCR_primers=fwd_name:  DeinoF,  rev_name:
Eubacterial
                     1107R"
rRNA<1..>839
                     /product="16S ribosomal RNA"
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      121 cagttaggaa tcttccccaa tggacgaaag tctgagggag cgacgccgcg tgagggatga
      181 aggttttcgg atcgtaaacc tctgaatcag ggacgaaaga cgcgtaagcg ggatgacggt
      241 acctgagtaa tagcaccggc taactccgtg ccagcagccg cggtaatacg gagggtgcaa
      301 gcgttacccg gaatcactgg gcgtaaaggg cgtgtaggcg gtacgctaag tctgacttta
      361 aagaccgggg ctcaaccccg gaagtgggtt ggatactggc gtgctggacc tctggagaga
      421 gaaccggaat tcctggtgta gcggtggaat gcgtagatac caggaggaac accgatggcg
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      601 catgggcgaa gctaacgcga taaacgtacc gcctgggaag tacggccgca aggttgaaac
      661 tcaaaggaat tgacgggggc ccgcacaagc ggtggagcat gtggtttaat tcgaagcaac
      721 gcgaagaacc ttaccaggtc ttgacatcca cagaaccttc cagagatggg agggtgccct
      781 tcggggaact gtgagacagg tgcggcatgg ctgtcgtcag ctcgtgtcgt gagatgttg
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Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
HQ738633.1	Uncultured Deinococcus sp. clone G10 16S ribosomal RNA gene, partial seq	1557	1557	100%	0.0	100%	
Y13042.1	D.murrayi 16S rRNA gene, isolate RSG-1.2	1434	1434	99%	0.0	98%	
HQ738632.1	Uncultured Deinococcus sp. clone G4 16S ribosomal RNA gene, partial sequ	1428	1428	99%	0.0	98%	
Y13043.1	D.murrayi 16S rRNA gene, isolate RSPS-7a	1415	1415	99%	0.0	97%	
NR_026416.1	Deinococcus murrayi DSM 11303 16S ribosomal RNA, partial sequence >emb	1406	1406	99%	0.0	97%	
HM299723.1	Uncultured bacterium clone ncd763f02c1 16S ribosomal RNA gene, partial s	1349	1349	99%	0.0	96%	
HM299639.1	Uncultured bacterium clone ncd762d09c1 16S ribosomal RNA gene, partial s	1339	1339	99%	0.0	96%	
AB264133.1	Deinococcus sp. TDMA-21 gene for 16S rRNA, partial sequence	1301	1301	99%	0.0	95%	
AY905382.1	Uncultured Deinococcus sp. clone V11-462 16S ribosomal RNA gene, partia	1301	1301	99%	0.0	95%	
NR_040934.1	Deinococcus aerius strain TR0125 16S ribosomal RNA, partial sequence >db	1288	1288	99%	0.0	94%	
JF418012.1	Bacterium EA10-69 16S ribosomal RNA gene, complete sequence	1286	1286	99%	0.0	94%	
GQ130015.1	Uncultured bacterium clone GI8-sp-L20 16S ribosomal RNA gene, partial se	1282	1282	99%	0.0	94%	
EU704886.1	Uncultured Deinococcus sp. clone 1P-1-M01 16S ribosomal RNA gene, parti	1282	1282	99%	0.0	94%	

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LOCUS       HQ738633                 843 bp    DNA     linear   ENV
09-MAR-2011
DEFINITION  Uncultured Deinococcus sp. clone G10 16S ribosomal RNA
gene,
partial sequence.
ACCESSION   HQ738633
VERSION     HQ738633.1  GI:325296108
KEYWORDS    ENV.
SOURCE      uncultured Deinococcus sp.
ORGANISM  uncultured Deinococcus sp.
            Bacteria; Deinococcus-Thermus; Deinococci; Deinococcales;
Deinococcaceae; Deinococcus; environmental samples.
REFERENCE   1  (bases 1 to 843)
AUTHORS   Chaturvedi,R. and Archana,G.
 TITLE       Development of a heminested polymerase chain reaction
            protocol for the detection of Deinococcus species from
            environmental samples
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 843)
  AUTHORS   Chaturvedi,R. and Archana,G.
  TITLE     Direct Submission
  JOURNAL   Submitted (20-DEC-2010) Department of Microbiology and
             Biotechnology  Centre,  M.S.  University  of  Baroda,
            Sayajigunj,Vadodara, Gujarat 390002, India
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source          1..843
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                     /mol_type="genomic DNA"
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                     /environmental_sample
                     /country="India"
                     /note="PCR_primers=fwd_name:  DeinoF,  rev_name:
Eubacterial
                     1107R"
rRNA<1..>843
                     /product="16S ribosomal RNA"
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121 cagcagttag gaatcttccc caatggacga aagtctgagg gagcgacgcc gcgtgaggga
181 tgaaggtttt cggatcgtaa acctctgaat cagggacgaa agacgcgtta tgcgggatga
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421 gagagaaccg gaattcctgg tgtagcggtg gaatgcgtag ataccaggag gaacaccgat
481 ggcgaaggca ggttcttgga cagaaggtga cgctgaggcg cgaaagtgtg gggagcgaac
541 cggattagat acccgggtag tccacaccct aaacgatgta cgttggctta tggcgggatg
601 ccgtcatggg cgaagctaac gcgataaacg taccgcctgg ggagtacggc cgcaaggttg
661 aaactcaaag gaattgacgg gggcccgcac aagcggtgga gcatgtggtt taattcgaag
721 caacgcgaag aaccttacca ggtcttgaca tcccaagaac ccctgagaga tcaggggtg
781 cccttcgggg aacttggaga caggtgctgc atggctgtcg tcagctcgtg tcgtgagatg
841 tgc
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Appendix III: 16S rRNA gene sequence of the radiation resistant bacterial isolates.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
HQ738629.1	Deinococcus sp. Grk2 16S ribosomal RNA gene, partial sequence	2676	2676	100%	0.0	100%	
CP002536.1	Deinococcus proteolyticus MRP, complete genome	2532	7598	98%	0.0	99%	
NR_026400.1	Deinococcus proteolyticus MRP 16S ribosomal RNA, partial sequence >emb|	2521	2521	98%	0.0	98%	
DQ003311.1	Deinococcus sp. 14 pro 16S ribosomal RNA gene, partial sequence	2416	2416	96%	0.0	98%	
DQ683348.1	Deinococcus piscis strain 3ax 16S ribosomal RNA gene, partial sequence	2381	2381	98%	0.0	97%	
NR_026402.1	Deinococcus radiophilus strain DSM 20551 16S ribosomal RNA, partial seque	2143	2143	98%	0.0	94%	
DQ664256.1	Deinococcus sp. IMCC1711 16S ribosomal RNA gene, partial sequence	2095	2095	98%	0.0	93%	
JF240719.1	Uncultured bacterium clone ncd2806g12c1 16S ribosomal RNA gene, partial	2025	2025	91%	0.0	94%	
JF181539.1	Uncultured bacterium clone ncd2095g07c1 16S ribosomal RNA gene, partial	2025	2025	91%	0.0	94%	
JF181385.1	Uncultured bacterium clone ncd2093g07c1 16S ribosomal RNA gene, partial	2025	2025	91%	0.0	94%	
JF181365.1	Uncultured bacterium clone ncd2093e08c1 16S ribosomal RNA gene, partial	2025	2025	91%	0.0	94%	
JF177724.1	Uncultured bacterium clone ncd2064h07c1 16S ribosomal RNA gene, partial	2025	2025	91%	0.0	94%	

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LOCUS       HQ738629          1449 bp    DNA     linear   BCT 09-MAR-
2011
DEFINITION     Deinococcus   sp.   Grk2   16S   ribosomal   RNA   gene,
partialsequence.
ACCESSION   HQ738629
VERSION     HQ738629.1  GI:325296104
KEYWORDS
SOURCE      Deinococcus sp. Grk2
ORGANISM    Deinococcus sp. Grk2
            Bacteria; Deinococcus-Thermus; Deinococci; Deinococcales;
Deinococcaceae; Deinococcus.
REFERENCE   1  (bases 1 to 1449)
AUTHORS     Chaturvedi,R. and Archana,G.
TITLE              Development   of   a   heminested   polymerase   chain
            reactionprotocol for the detection of Deinococcus species
            from environmental samples
JOURNAL     Unpublished
REFERENCE   2  (bases 1 to 1449)
AUTHORS     Chaturvedi,R. and Archana,G.
TITLE       Direct Submission
JOURNAL    Submitted (20-DEC-2010) Department of Microbiology and
            Biotechnology   Centre,   M.S.   University   of   Baroda,
Sayajigunj,
            Vadodara, Gujarat 390002, India
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                     /isolation_source="Greater Rann of Kutch"
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rev_name:
                     Eubacterial 1541R"
rRNA<1..>1449
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121 gggctaatac aggatgcgca gtatcgctgt ggcgatacta caaagattta tcgcttcggg
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301 ggaggcagca gttaggaatc ttccacaatg ggcgaaagcc tgatggagcg acgccgcgtg
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661 caatggcgaa ggcaagttac tggacagaag gtgacgctga ggcgcgaaag tgtgggggc
721 gaaccggatt agatacccgg gtagtccaca ccctaaacga tgtacgttgg tctagcgcag
781 gatgctgtgt tggacgaagc taacgcgata aacgtaccgc ctgggaagta cggccgcaag
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901 gaagcaacgc gaagaacctt accagtcctt gacatgcacg gaacgcgctg gaagcagcgc
961 ggtgcccttc ggggaaccgt gacacaggtg ctgcatggcg tcgtcggctc gtgtcgtgag
1021 atgttgggtt aagtcccgca acgagcgcaa cccttgcctt tagttgccag catttggttg
1081 ggcactctag agggactgcc tgtgaaagca ggaggaaggc ggggatgacg tctagtcagc
1141 atggtcctta cggtctgggc tacacacgtg ctacaatggc cggtacaacg cgcagcgaac
1201 ttgtgagagt aagcgaatcg ctaaaagccg gcctcagttc agattggagt ctgcaactcg
1261 actccatgaa gtggaatcgc tagtaatcgt gggtcagcat accgcggtga atacgttccc
1321 gggccttgta cacaccgccc gtcacaccat gggagtagat tgcagctgaa accgctggga
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1441 aaaggcggc
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Accession	Description	Max score	Total score	Query coverage	E value	Max ident	Links
HQ876598.1	Deinococcus sp. Grk4 16S ribosomal RNA gene, partial sequence	2774	2774	100%	0.0	100%	
JN082273.1	Deinococcus ficus strain cp4502 16S ribosomal RNA gene, partial sequence	2634	2634	97%	0.0	99%	
JN082263.1	Deinococcus ficus strain cp02 16S ribosomal RNA gene, partial sequence	2634	2634	97%	0.0	99%	
NR_043282.1	Deinococcus ficus strain CC-FR2-10 16S ribosomal RNA, partial sequence >	2623	2623	96%	0.0	99%	
HQ738630.1	Deinococcus sp. Grk5 16S ribosomal RNA gene, partial sequence	2551	2551	97%	0.0	98%	
DQ223542.2	Deinococcus sp. X2 16S ribosomal RNA gene, complete sequence	2545	2545	97%	0.0	98%	
DQ223542.2	Deinococcus sp. G8 16S ribosomal RNA gene, partial sequence	2545	2545	97%	0.0	98%	
DQ003135.1	Deinococcus mumbaiensis strain CON-1 16S ribosomal RNA gene, partial se	2532	2532	94%	0.0	99%	
HQ858011.1	Deinococcus sp. NIO-201103 16S ribosomal RNA gene, partial sequence	2429	2429	89%	0.0	99%	
HM291155.1	Uncultured bacterium clone ncd690c01c1 16S ribosomal RNA gene, partial s	2418	2418	88%	0.0	99%	
NR_026399.1	Deinococcus grandis strain DSM 3963 16S ribosomal RNA, partial sequence	2274	2274	97%	0.0	95%	
HQ144169.1	Deinococcus sp. R14 16S ribosomal RNA gene, partial sequence	2242	2242	85%	0.0	98%	

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LOCUS       HQ876598                1502 bp    DNA     linear   BCT
05-MAR-2011
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ACCESSION   HQ876598
VERSION     HQ876598.1  GI:324435606
KEYWORDS    .
SOURCE      Deinococcus sp. Grk4
ORGANISM    Deinococcus sp. Grk4
            Bacteria; Deinococcus-Thermus; Deinococci; Deinococcales;
Deinococcaceae; Deinococcus.
REFERENCE   1  (bases 1 to 1502)
AUTHORS    Chaturvedi,R. and Archana,G.
TITLE       Development of a heminested polymerase chain reaction
           protocol          for the detection of Deinococcus species
           from environmental samples
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 1502)
  AUTHORS   Chaturvedi,R. and Archana,G.
  TITLE     Direct Submission
  JOURNAL   Submitted (09-JAN-2011) Department of Microbiology and
            Biotechnology Centre, The M.S. University of Baroda,
            Sayajigunj, Vadodara, Gujarat 390 001, India
FEATURES             Location/Qualifiers
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                     /country="India: Gujarat"
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rev_name:
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121 cccaagtcgc ggataactgg ccgaaaggtc agctaatacg tgatgtgatg ccccttttct
181 ggagggtcat taaaggttta ctgcttgggg atggggttgc gttccatcag ctagttggcg
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TITLE Development of a heminested polymerase chain reaction protocolfor
the detection of Deinococcus species from environmental samples
JOURNAL Unpublished
REFERENCE 2 (bases 1 to 1464)
AUTHORS Chaturvedi,R. and Archana,G.
TITLE Direct Submission
JOURNAL Submitted (20-DEC-2010) Department of Microbiology and Biotechnology Centre, M.S. University of Baroda, Sayajigunj,
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Know how to solve every problem that has ever been solved."
- Richard Feynman

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Summary

Because all of biology is connected, one can often make a breakthrough with an organism that exaggerates a particular phenomenon, and later explore the generality

The genus *Deinococcus* is represented by members that are extremely resistant to ionising as well as non-ionising radiation, desiccation, H_2O_2 and several mutagenic agents as mitomycin C and hence are aptly described as "polyextremophiles". The isolation of these organisms often employs ionising radiation as selective pressure that eliminates the radiation sensitive population, obliterating any information regarding the comparative distribution of this group in natural habitats.

A molecular method based on 16S rRNA gene was developed for easy detection and understanding the deinococcal diversity from different environmental samples. An oligonucleotide sequence corresponding to positions 202-222 (according to the numbering in *D.indicus* 16SrRNA gene), is present in > 70% of the total deinococcal spesies listed at the RDP and has negligible possibility of being found in other taxonomic groups. Employing PCR primer corresponding to this sequence, a two step hemi–nested PCR protocol for the detection of deinococci from mixed microbial communities was developed. Under optimised conditions, this procedure was highly specific and had sensitivity to detect 1pg of the deinococcal 16S rDNA obtained from the first round of PCR while 16SrDNA of none of the other bacteria tested was detected. Using this method, the presence of deinococci was detected in 10 different soil samples from diverse ecological settings in India. To comprehend the deinococcal diversity of the environmental samples, a group specific 16S rDNA clone library was prepared from representative samples. Clones from the CRC sample showed maximum identity to *D. radiopugnans* while clones from GRK represented phylotypes showing maximum homology to *D. murrayi* and *D. hopeinsis*. The hemi – nested PCR approach was extended to develop a three step PCR-DGGE protocol for obtaining fingerprints of the deinococcal community in the environmental samples. A rich diversity of deinococci was detected in the samples, which was greater than that revealed by the clone library. Sequence analysis of the DGGE bands demonstrated the dominance of *D. radiopuganans* obtained from different environments. *D. radiodurans*, *D. deserti*, *D. proteolyticus*, and *D. murrayi* were also represented amongst the other DGGE band sequences. Culturing of radiation resistant bacteria from these samples lead to isolation of only a few deinococcal pure cultures indicating that the clonal diversity was from unculturable deinococci. Three novel radiation resistant isolates were obtained from GRK samples and were ascertained to be deinococci with hemi-nested PCR approach. 16S rRNA gene sequence analysis of the isolates revealed Grk2 was similar to *D. proteolyticus* while Grk4 and Grk5

showed maximum similarity to *D. ficus*. The newly isolated strains were catabolically more versatile than *D. radiodurans* R1 (DR1) and showed broader spectrum of carbohydrate utilisation as compared to the type strain DR1 indicative of robust physiology. Some possessed better resistive properties than DR1. Considering the potential use of radiation resistant bacteria for bioremediation of the nuclear waste sites, tolerance of the new the isolates to heavy metals commonly found at the radioactive waste sites, was also analysed and some isolates showed significantly better metal tolerance than DR1.

Most bacteria are reported to be more resistant to external stress during the stationary phase. On the contrary we observed that DR1 was more sensitive to Cd^{2+} during the stationary phase and the log phase cells of DR1 exhibited a D_{50} three times as much as the stationary phase culture. The sensitivity of the stationary phase culture to Cd^{2+} was rescued by the addition of 100μM Mn^{2+}, which then exhibited a D_{50} comparable to the log phase culture. Cd^{2+} induced stasis was observed for the stationary phase culture for 48h. During recovery from Cd^{2+} induced stasis several morphological changes were observed, most profound changes being, after 3h and 6h post recovery.

The reactive oxygen species (ROS) induced by Cd^{2+} was demonstrated to produce in DR1. Carbonylated proteins which are indicators of the oxidative damage were found to increase in a dose dependent manner in DR1 exposed to Cd^{2+}. Lipid damage was in coherence with the ROS produced. Both superoxide dismutase (SOD) and catalase enzymes, which are regarded as first line of defence against the oxidative stress, were inhibited in the presence of Cd^{2+}. It can be assumed the loss of catalase activity could be because of the Fe displacement by Cd^{2+} by from the active as catalytic site of catalases from DR1 *kat*A, known to be heme catalase. *sod* A^- mutant of DR1, defective in SOD, was 5 times as sensitive to Cd^{2+}as compared to the wild type. In contrast to the wild type, Mn^{2+} aggravated Cd^{2+} toxicity to the *sod* A^-.

Pre-exposure to sub-lethal concentration of H_2O_2 provided cross-resistance Cd^{2+}. Conversely, DR1 cells grown in presence of Cd^{2+} decimate the catalase activity and reduce the ability of the culture to withstand H_2O_2. Similarly, prior exposure to UV sensitises DR1 culture for better recovery in presence of Cd^{2+} indicating a possible role of the UV repair pathway in tolerance to Cd^{2+} in DR1. The *rec* A^- mutant of DR1 was found to be 4 fold sensitive to Cd^{2+} as compared to the wild type though the

addition of Mn^{2+} didn't alter the tolerance to Cd^{2+} for the *rec* A^- mutant. The *lac* Z reporter gene under *rec*A promoter exhibited a dose dependent increase in beta-galactosidase activity with Cd^{2+} exposure, indicating a possible role of *rec*A in Cd^{2+} tolerance in DR1. PprI, known to regulate both SOD and *rec* A in DR1, when mutated showed sensitivity to Cd^{2+} exhibiting a D_{50} less than either *rec* A^- or *sod* A^-.

Proteomic analysis of the DR1 grown under log phase, stationary phase and log phase amended with Cd^{2+} or Mn^{2+} indicate that there exists an overall large amount of similarity amongst the proteins expressed during stationary phase, Mn^{2+} induced and Cd^{2+} induced cultures of DR1, therefore it can be concluded that exogenous Mn^{2+} can also exert stressful conditions in DR1. No proteins were detected exclusively for both stationary phase cultures and Cd^{2+} affected cells indicating that DR1 may have more generalised response to combat the Cd^{2+} stress and no unique mechanism to combat Cd^{2+}. Metalloproteome analysis of the DR1 grown under log phase, stationary phase and log phase amended with Cd^{2+} or Mn^{2+} was done using immobilised metal affinity chromatography. Mn^{2+} and Cd^{2+} binding proteome in DR1 under all conditions bear a significant resemblance. Although there were significant differences obtained for Mn^{2+} binding proteome, but the Cd^{2+} binding proteome was similar under all conditions examined.

Construction of recombinant strains of DR1 that can survive high metal concentrations at the radioactive waste sites and subsequent co-transformation of such strains with metabolic genes for mineralization of toxic hydrocarbon and metal can enhance the applicability of DR1 for bioremediation at the nuclear waste sites. Cloning and expression of natural metallothionein gene, *smt* A and synthetic phytochelatin gene, *ec* 20 was carried out in DR1. The synthetic phytochelatin was synthesised by overlap extension PCR using synthetic oligonucleotides. *ec*20 and *smt* A were expressed individually in DR1 under the effect of *gro*EL promoter in the shuttle vector, pRADZ3 cloned in place of the *lacZ* fragment. The transformant DR1 (pRAD-EC) exhibited, 1.5 fold higher tolerance to Cd^{2+} than the control and accumulated 1.21 fold greater Cd^{2+} as opposed to the control. Heterologous expression of natural metallothionein gene, *smt* A, in DR1 imparted the transformant, superior tolerance to Cd^{2+} wherein DR1 (pRAD-*smt*A) amassed 2.5 fold greater Cd^{2+} than DR1-EC. Addition of cysteine enhanced the growth of the DR1 strains harbouring the metallothionein however it did not translate into efficient metal accumulation.

Briefly, the highlights/achievements of the present work can be summarised as follows

- A *Deinococcus* specific primer was demonstrated to be useful for group specific PCR and studying deinococcal diversity by different culture-independent approaches
- Three new deinococcal isolates have been obtained from Great Rann of Kutch, an Indian salt desert. These are catabolically more versatile and more robust in terms of their resistive properties than the most popular strain, *D. radiodurans* R1.
- Deinococcal diversity, in terms of their DGGE fingerprints, has been reported for the first time. Distinct communities have been observed in different Indian ecological settings and preponderance of specific species observed.
- Several new deinococcal 16S rRNA gene sequences from uncultured as well as the three new isolates are reported from different geographic regions of India, some locations for the first time. Presence of deinococci in contaminated environments (Cr contaminated and petrol contaminated samples) has been detected; however their isolation was not possible.
- A detailed study of the effects of Cd, a toxic metal, in DR1 has been undertaken, particularly to understand growth phase dependent sensitivity as well as Mn-Cd interactions in this strain. The studies have provided new insights about the oxidative stress imposed by Cd^{2+} in DR1 and some aspects of its dependence on other factors both genetic and physiological
- The expression of synthetic phytochelatin and a natural heterologous metallothionein gene in DR1 is reported to bring about modest increase in Cd^{2+} tolerance.

Presentations and Publications

Do what you can, with what you have, where you are.

-Theodore Roosevelt

Publication

1. **Chaturvedi, R**., G. Archana. Novel 16S rRNA primers targeting the genus *Deinococcus* spp. and its application to assess the diversity of deinococcal populations in environmental samples. ***J. Microbiol. Methods***. **90:** 197- 205.

Posters presented

1. **Chaturvedi, R**., R. Madhumitha., Desai, A.J. and G.Archana. Effect of Manganese on Cadmium toxicity in ionizing radiation-resistant bacteria. Poster presented at 45th **Annual meet of the Association of Microbiologist of India**, Osmania University, Hyderabad, and 7th December -10th December, 2005.
2. **Chaturvedi, R**., Das, S., Desai, A.J. and G.Archana. Development of a hemi-nested PCR-based method for the detection of *Deinococcus* spp. Poster presented at **75th Annual meeting of Society of Biological Chemists** conference held at JNU, New Delhi, and 8th December -11th December, 2006.