A DISSERTATION ON

Overexpression of DR_1143 protein and its biochemical study

SUBMITTED TO THE
DEPARTMENT OF BIOENGINEERING
FACULTY OF ENGINEERING & INFORMATION TECHNOLOGY
INTEGRAL UNIVERSITY, LUCKNOW

IN PARTIAL FULFILLMENT
FOR THE
B.TECH-M.TECH DUAL DEGREE
IN BIOTECHNOLOGY

BY
Anam Khan
B.Tech-M.Tech Dual Degree Biotechnology (X Semester)
Roll No: 1801101002

UNDER THE SUPERVISION OF
Dr. Anaganti Narasimha
Scientific Officer-F
Molecular Biology Division
Bhabha Atomic Research Centre
Mumbai-400085

INTEGRAL UNIVERSITY, DASAULI, KURSI ROAD
LUCKNOW- 226026

DECLARATION FORM

I, **Anam Khan**, a student of **B.Tech-M.Tech Dual Degree Biotechnology** (V Year/ X Semester), Integral University have completed my six months dissertation work entitled "**Overexpression of DR_1143 protein and its biochemical study**" successfully from **Molecular Biology Division, Bhabha Atomic Research Centre, Mumbai** under the able guidance of **Dr. Anaganti Narasimha.**

I, hereby, affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reported in this study are genuine and authentic.

Anam Khan

Dr. Reena Vishvakarma
(Course Coordinator)

सत्यमेव जयते

भारत सरकार
GOVERNMENT OF INDIA

भाभा परमाणुअनुसंधानकेंद्र
BHABHA ATOMIC RESEARCH CENTRE

Molecular Biology Division

CERTIFICATE

This is to certify that **Ms. Anam Khan**, B.Tech-M.Tech Dual Degree Biotechnology student of Integral University, Lucknow has carried out the project entitled **"Overexpression of DR_1143 protein and its biochemical study"** under the supervision of **Dr. Anaganti Narasimha (Scientific Officer–F)** in our laboratory in the Molecular Biology Division, Bhabha Atomic Research Centre, Mumbai from 15th February 2023 to 15th August 2023.

The work carried out is going to be submitted to the Integral University, Lucknow for the partial fulfillment of B.Tech-M. Tech Dual Degree in Biotechnology.

Date:

Place: BARC, Mumbai

Dr. Bhakti Basu
Scientific Officer – G
Head, Stress Genome &
Proteome group

Dr. Anaganti Narasimha
Scientific Officer – F

INTEGRAL UNIVERSITY
LUCKNOW - INDIA
Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

DECLARATION OF CONFIDENTIALITY

We hereby declare that the project **"Overexpression of DR_1143 protein and its biochemical study"** is a record of research work carried out by **Ms. Anam Khan** under the guidance of **Dr. Anaganti Narasimha (Scientific Officer-F)**, Molecular Biology Division, Bhabha Atomic Research Centre, Trombay, Mumbai-400085.

This project is submitted to the **Department of Bioengineering, Integral University, Lucknow** as academic mandatory requirement for the partial fulfilment of B.Tech-M.Tech Dual Degree Biotechnology, and no part of this research work will be published in any national or international journals without the permission of concerned BARC authorities. It will not be presented in any symposium or conferences. We also assure you that no part of this experimental data will be used for any personal benefits.

Date:

Place:

Ms. Anam Khan
B.Tech-M.Tech
Dual Degree Biotechnology

Dr. Alvina Farooqui
Professor and Head
Department of Bioengineering
Faculty of Engineering & Information Technology

INTEGRAL UNIVERSITY
LUCKNOW - INDIA
Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

CERTIFICATE BY INTERNAL ADVISOR

This is to certify that **Anam Khan,** a student of **B.Tech-M.Tech Dual Degree Biotechnology** (V Year/X Semester), Integral University has completed her six months dissertation work entitled **"Overexpression of DR_1143 protein and its biochemical study"** successfully. She has completed this work at **Molecular Biology Division, Bhabha Atomic Research Centre, Mumbai** under the guidance of **Dr. Anaganti Narasimha, Scientific Officer- F, Molecular Biology Division, Bhabha Atomic Research Centre, Mumbai**. The dissertation was a compulsory part of her **B.Tech-M.Tech Dual Degree Biotechnology.**

I wish her good luck and a bright future.

Dr. Adnan Ahmad
Assistant Professor
Department of Bioengineering
Faculty of Engineering & Information Technology

INTEGRAL UNIVERSITY
LUCKNOW - INDIA
Phone No.: +91(0522) 2890812, 2890730, 3296117, 6451039, Fax No.: 0522-2890809
Kursi Road, Lucknow-226026 Uttar Pradesh (INDIA)

TO WHOM IT MAY CONCERN

This is to certify that **Anam Khan,** a student of **B.Tech-M.Tech Dual Degree Biotechnology** (V Year/ X Semester), Integral University has completed his/her six months dissertation work entitled **"Overexpression of DR_1143 protein and its biochemical study"** successfully. She has completed this work from **Molecular Biology Division, Bhabha Atomic Research Centre, Mumbai** under the guidance of **Dr. Anaganti Narasimha**. The dissertation was a compulsory part of his/her **B.Tech-M.Tech Dual Degree Biotechnology.**

I wish her good luck and a bright future.

Dr. Alvina Farooqui
Professor and Head
Department of Bioengineering
Faculty of Engineering & Information Technology

Acknowledgement

It gives me great joy to be able to express my gratitude to everyone for their support and encouragement in making this internship a success. First and foremost, I must acknowledge and thank Allah for blessing and guiding me throughout this period.

I would like to express my gratitude to **Dr. Bhakti Basu (SO/G)**, **Head**, **Stress Genomics and Proteomics Group**, **BARC** for her constant support and motivation throughout the project.

I am immensely grateful to my supervisor **Dr. Anaganti Narasimha (SO/F)**, **BARC** for his guidance, expertise, and unwavering support. His insightful feedback, patience, and encouragement were instrumental in shaping the direction of this research and refining my ideas. I am truly fortunate to have had his mentorship.

I express my profound gratitude to Dr. Aman Kumar Ujaoney (SO/G) and Mr. Mahesh Kumar Padwal (SO/D) for supporting and helping me whenever needed. I would like to extend my gratitude to Mrs. Pratiksha Dani (TO/D) and Mr. Gaurav Wilton (Foreman/B) for always helping me with all secondary lab work and procuring materials required for the project.

I would like to sincerely thank **Dr. Alvina Farooqui**, **Professor and Head**, Department of Bioengineering, Faculty of Engineering and Information Technology, Integral University, Lucknow for permitting me to complete my project work at such a prestigious institution.

Also, I would like to extend the acknowledgement to the **Chancellor**, **Prof. S. W. Akhtar**, the **Pro Chancellor**, **Dr. Syed Nadeem Akhtar**, the **Vice Chancellor**, **Prof. Javed Musarrat**, and **Prof. (Dr.) Tahseen Usmani**, **Dean**, **Integral University** for their support and acceptance in undertaking this project at BARC. I am gratefully indebted to my **Internal Advisor**, **Dr. Adnan Ahmad** for his constant support and guidance throughout the project. In addition, I would like to thank **Dr. Reena Vishvakarma, Course Coordinator**, **Dr. Khwaja Osama**, **U.G. Coordinator**, **Dr. Roohi**, **P.G**.

Coordinator, the faculty members of the department and the support staff, for their cooperation, affection, and engagement during my project pursuit.

Last but not least, I extend my heartfelt thanks to my family and friends for their unwavering love, encouragement, and belief in my abilities.

Date: 15th August 2023 ANAM KHAN

Index

List of figures

List of tables

List of abbreviations

APS: Ammonium Persulphate

ddUTP: 2',3'-Dideoxyuridine-5'-triphosphate

DIG: Digoxigenin

dNTP: deoxyNucleoside Triphosphate

DOC: Deoxycholate

DR: *Deinococcus radiodurans*

DSB: Double Strand breakage

E. coli: *Escherichia coli*

EDTA: Ethylene Diamine Tetra Acetic Acid

ESDSA: Extended synthesis-dependent strand annealing

Gy: Gray

kbp: kilobase pair

KEGG: Kyoto Encyclopedia of Genes and Genomes

LB: Luria Bertani

mM: Millimolar

M: Molar

ml: Millilitre

µl: Microlitre

MMS: Methyl Methanesulfonate

ng: Nanogram

NBT- BCIP: nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyl phosphate

NHEJ: Non- Homologous End Joining

OD: Optical density

PAGE: Polyacrylamide Gel Electrophoresis

PCR: Polymerase Chain Reaction

Phyre: Protein Homology/AnalogY Recognition Engine

pM: Picomolar

RPA: Replication protein A

priB: Primosomal replication protein N

RPM: Revolution per Minute

RCF: Relative Centrifugal Force

SASPs: Small acid-soluble proteins

SSA: Single Strand Annealing

SDS: Sodium Dodecyl Sulphate

SSB: Single Strand breakage

TBE: Tris Borate EDTA

TCA: TrichloroAcetic Acid

TEMED: Tetramethylethylenediamine

TGY: Tryptone Glucose Yeast

CHAPTER 1

INTRODUCTION

According to The Guinness Book of World Records, *Deinococcus radiodurans* R1, a member of the *Deinococcaceae*, is the most resilient bacterium ever discovered.It is one of the species with the highest radiation resistance and is referred to as a polyextremophile as it can endure cold, dehydration, vacuum, and acid (Deweerdt S, 2002). Its name is derived from the Greek words “deinos” and “coccus”, which mean “a grain or berry” and “strange or unusual”, respectively and the Latin words “radius” and “durare”, which imply “radiation surviving” (Slade D and Radman M, 2011). It has also earned the nickname “Conan the Bacterium” due to its capacity to withstand extremely high radiation levels (Huyghe P, 1998). It is one of the few creatures capable of withstanding radiation doses so high that the genome is broken up into hundreds of tiny fragments (Zahradka *et al.,* 2006). Compared to *E. coli* and humans, it is 30 times and 1,000 times resilient to ionising radiation respectively (Harsojo *et al*., 1981).

D. radiodurans is unparalleled in its capacity to exhibit extreme resistance against DNA-damaging substances such as ionising radiation (X- rays and Gamma rays) that generate double and single stranded breaks, non-ionizing radiations (UV rays) that cause pyrimidine dimer formation, chemicals such as hydrogen peroxide, methyl methanesulfonate (MMS), nitrous acid, hydroxylamine, that can cause base and nucleotide damages (Sweet DM and Moseley BE, 1974). Proteins, lipids, nucleic acids, and carbohydrates are damaged by ROS produced by desiccation and ionising radiation, which also cause DSBs (Slade D and Radman M, 2011). A very effective DNA repair system is the mainstay of its resilience (Setlow JK and Duggan DE, 1964). Any organism exposed to 5 kGy of IR develops hundreds of DSBs in its genome, and Deinococcus is no exception. But within 3–4 hours, *Deinococcus* repairs them, splicing overlapping pieces of DNA to form whole chromosomes, and the cells soon return to their regular growth (Basu B and Apte SK, 2012).

These factors have made *D. radiodurans* a top choice for studying the DNA destruction , repair mechanisms and practical applications, such as stabilising and cleaning up regions contaminated radioactively (Makarova KS *et al*., 2001). Additionally, the antioxidation protection mechanisms in *D. radiodurans* may offer approaches to delay ageing and prevent cancer. Extreme ionising radiation resistance in D. radiodurans may also shed light on cytotoxic and radiotherapy treatment resistance. Due to this, *D. radiodurans*

research as an experimental organism for antioxidant resistance has a lot of promise and will continue to have an important influence on medicine and public health on a global scale (Slade D and Radman M, 2011).

The main mechanism of the bacteria to survive such high doses and DNA damage is still not known precisely. Different hypotheses and possibilities have suggested that it combines and uses many physiological tools for the radioresistance which are tightly co-ordinated (Blasius *et al*., 2013). *D. radiodurans* responds to stresses by upregulating the expression of many genes, only a small number of which have been detected (Tanaka M *et al*., 2004). The *Deinococcaceae* has 206 or 230 distinct proteins. Only 5 DNA repair-related proteins, DdrB, DdrC, DdrD, PprA, and DdrO, have been identified, of which DdrB and PprA have been characterised (DeGroot A *et al*., 2009).

There are some uncharacterized genes present in the genome of *Deinococcus radiodurans* whose function is still unknown. They might be playing a role in the DNA repair mechanism. One such gene is DR_1143 whose function is still unknown but it gets upregulated in various stress conditions suggesting its involvement in the radiation resistance of the organism (Anaganti N *et al.,* 2016).

Aim: <u>Overexpression of DR_1143 protein and its biochemical study</u>

Objectives:

1. **Cloning, overexpression of DR_1143 protein and protein purification.**
2. **Biochemical studies in terms of DNA-protein binding by EMSA (Electrophoretic Mobility Shift Assay).**

CHAPTER 2

LITERATURE REVIEW

2.1 About the organism-*Deinococcus radiodurans*

Deinococcus radiodurans is a Gram-positive, non-motile, non sporulating, nonpathogenic bacteria (White O *et al*., 1999). It was initially isolated from spoiled canned meat after being exposed to X-rays. It uses oxygen to get energy from organic materials in its environment and is an obligate aerobic chemoorganoheterotroph (Battista JR *et al*., 1997). The colonies range in colour from pink to red due to carotenoid pigment deinoxanthin and are smooth and convex with a cell diameter ranging from 1.5 to 3.5 μm (Murray RG, 1986). It takes roughly 100 minutes for a cell to double, and colonies need three days to develop properly when grown conventionally at 32° C in TGY media with aeration (Blasius M *et al*., 2013).

The thermal limit of this mesophile is above 39 °C. The cells are vegetative and can be cultured easily. Tetrads are produced when cells divide alternately in two planes by binary fission; its great degree of radiation resistance is thought to be caused by its tetrad structure (Makarova KS *et al*., 2001). The cells are totally competent and can efficiently take up the free DNA due to its transformational ability (Moseley BE and Setlow JK 1968). *D. radiodurans* is the only species in its genus to possess this characteristic, making it the most widely studied organism. Additionally, the bacteria is susceptible to antibiotics which inhibit the production of RNA, proteins, and cell walls (Hawiger J and Jeljaszewicz J, 1967). Images of *Deinococcus radiodurans* R1 under bright field, electron microscope, fluorescence microscope and nanoscale X-ray imaging are shown in **figure 1**.

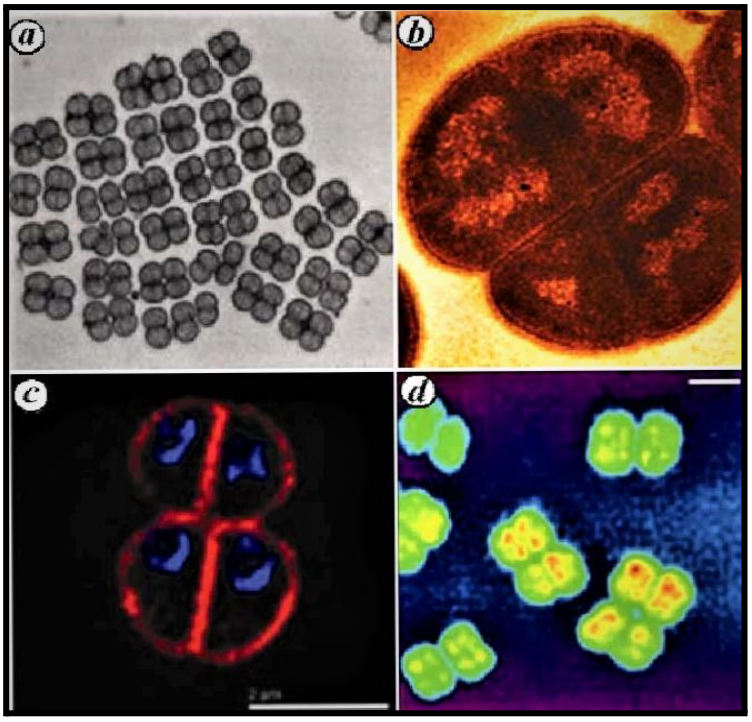


Figure 1. Photograph of *Deinococcus radiodurans* R1 under
(A) bright field (B) electron microscope
(C) fluorescence microscope (D) nanoscale X-ray imaging
(Source: Misra HS *et al.,* 2013**)**

2.1.1 History

Laboratory tests planted the historical seeds of *D. radiodurans*. At the Oregon Agricultural Experiment Station in Corvallis, Arthur W. Anderson made the discovery of *D. radiodurans* in 1956 (Makarova KS *et al*., 2007). High doses of gamma radiation were being used in tests to see if canned food could be sterilised. A dose of radiation was used to sterilise a can of meat, but the flesh rotted after being subjected to the radiation. The red bacteria were first noticed by Anderson, who later isolated them and eventually identified as *Deinococcus radiodurans* (Anderson AW, 1956).

2.1.2 Ecology

The *Deinococcaeace* family comprises mesophilic, thermophilic, and psychrophilic members which have ubiquitous ecological distribution. *Deinococcus* species can be found in a variety of habitats, including animal guts, hot springs, deserts, alpine settings, and Antarctica (Slade D and Radman M, 2011). Shortly after the isolation of *D. radiodurans* R1 in 1956, a second strain of *D. radiodurans* (SARK) was found as an air contaminant in an Ontario hospital (Makarova KS *et al*., 2001).

There hasn't been a thorough investigation of Deinococci's natural spread. Isolations have happened all across the world, with sites ranging from a variety of dry, nutrient-poor environments, such as weathered granite in a dry Antarctic valley, room dust, and irradiated medical equipment, as well as from places rich in organic nutrients like soil, animal faeces, and processed meat (White O et al., 1999). Recently, it has been suggested that adaptation may also occur in permafrost and other semi-frozen environments. Till date, 43 distinct *Deinococcus* species have been isolated, frequently through radiation selection (Slade D and Radman M 2011).

2.1.3 Classification (Phylogeny and Taxonomy):

It has been challenging to classify *Deinococcus radiodurans*. Due to the peptidoglycan in its cell wall, it is not an Archaebacteria, but it does have genes that are not present in Eubacteria. Based on its physical and physiological traits, it was given the name *Micrococcus radiodurans*. It was assigned to a new family, the *Deinococcaceae*, and given the name *Deinococcus radiodurans* since 16S rRNA research revealed that *M. radiodurans* belongs to a unique evolutionary group of bacteria (Raj HD *et al.,* 1960).

D. radiodurans was discovered to be phylogenetically related to *Thermus thermophilus* based on the study of 16S rRNA sequences. There is a significant megaplasmid homology between them. However, they also exhibit strikingly dissimilar phenotypes. *T. thermophilus* is radiation-sensitive, but *D. radiodurans* is a radiation-resistant bacteria (Holt JG *et al*., 1997).

Because its cell wall contains peptidoglycan, *D. radiodurans* is thought to belong in the domain and kingdom of bacteria **(table 1)**. The majority are mesophiles, and every

member is radiation resistant. The only other genus found within the class *Deinococci* in addition to *Deinococcus* is *Deinobacter.* The radiation-resistant bacteria *Deinobacter grandis,* which has a rod-shaped morphology rather than the conventional spherical cocci morphology characteristic of the genus *Deinococcus*, led to the creation of this new genus.

Recently, the *Trueperaceae* family was included in the *Deinococcus-Thermus* phylum. Another radioresistant bacterium of the *Deinococcus* lineage, *Truepera radiovictrix* is the most alkaliphilic organism and grows best at a temperature of 50°C (Albuquerque L *et al.,* 2005).

Kingdom	Bacteria
Phylum	Deinococcus- Thermus
Class	Deinococci
Order	Deinococcales
Family	*Deinococcaaceae*
Genus	*Deinococcus*
Species	*Deinococcus radiodurans*

Table 1: Classification of *D. radiodurans*

2.1.4 Genome

In 1999, the complete DNA sequence of *D. radiodurans* was published (Blasius M *et al*., 2008). The genome consists of two chromosomes, a megaplasmid, and a small plasmid **(table 2)**, yielding 3,284,123 bp (White O *et al*., 1999). Each cell may have up to 10 copies of the genome, which has 3,187 ORFs and a significant GC amount of 66.6% (Driedger AA, 1970). The number of genome copies in exponentially proliferating cells ranges from 4 to 10, depending on the growth stage and media conditions (Harsojo *et al.,* 1981).

In addition to being involved in cellular energy production, proteins encoded on chromosome II and the megaplasmid may be crucial for *D. radiodurans'* recovery from famine and/or desiccation. Megaplasmid genes that produce dNTPs or dNTP precursors are likely involved in the repair of damaged DNA (White O *et al*., 1999).

Molecule	Length (bp)	Average ORF length (bp)	Protein coding regions	GC-contents	Repeat content
Chromosome 1	26,48,615	740	90.80%	67.00%	1.80%
Chromosome 2	4,12,340	846	93.50%	66.70%	1.40%
Megaplasmid	1,77,466	802	90.40%	63.20%	9.20%
Plasmid	4,55,702	257	80.90%	56.10%	13.00%
Total	32,84,123	748	90.90%	66.60%	3.80%

Table 2: Genome features of *D. radiodurans*

2.1.5 Cell Structure

The chemistry and arrangement of the cell envelope are distinct. Despite being Gram-positive, it has a multilayered structure and lipid makeup comparable to Gram-negative bacteria (Lancy P and Murray RG, 1978). The cell envelope is made up of five layers totaling 150 nm in thickness: the compartmentalised layer, the inner layer, the cytoplasmic membrane, the peptidoglycan-containing holey layer made of mucopeptide, the inside layer, and the fragile soft layer (Work E and Griffiths H, 1968).

D. radiodurans have a unique fatty acid composition. Even-numbered, straight-chain, saturated fatty acids dominate in lipoproteins but the bacteria also has a considerate amount of odd-numbered, saturated, and monounsaturated fatty acids in lipoproteins, which are not typically seen in bacteria (Girard AE, 1971). The fluidity of membranes is enhanced by unsaturated fatty acids and they are also crucial for membrane dynamics during desiccation stress. *D. radiodurans* is distinguished by the absence of typical phospholipids found in other bacteria (Anderson R and Hansen K, 1985). Phosphoglycolipids with alkylamines as structural components make up 43% of membrane lipids (Slade D and Radman M, 2011).

2.1.6 Cell Division

Cells of *D. radiodurans* divide in two planes alternately (Murray RG *et al*, 1983). The cross wall parallel to the prior division is built when the following division begins (Thornley MJ *et al*., 1965). In contrast to other cocci, *D. radiodurans* cells divide by having two septa sweep across the cell from opposing sides to create a slit closure. The

newly formed septation starts before the septal curtains join, allowing communication between rapidly developing cells through four compartments (Murray RG *et al*., 1983).

The membrane of the cytoplasm and the layer of peptidoglycan play a role in septum formation during cell division. During growth, the microcolonies form a rectangular array (Driedger AA, 1970). This organised growth pattern persists for a minimum of five generations (Slade D and Radman M, 2011). Interestingly, X-ray fluorescence microprobe study has revealed that during cell division, Fe^{2+}ions accumulate at the dividing septum while Mn^{2+} ions concentrate at the nucleoid (Daly MJ *et al*., 2007).

2.1.7 Metabolic configuration

Organotrophic bacteria *D. radiodurans* requires a minimum of a carbon source, nicotinic acid, sulphur, nitrogen, and manganese (Makarova KS *et al*., 2007). It is a proteolytic bacteria which utilises amino acids as a preferred primary carbon source (He Y, 2009). Carbohydrates are probably imported via the megaplasmid-encoded phosphoenolpyruvate phosphotransferase pathway (Venkateswaran A *et al*., 2000). The bacterium resynthesizes all of the depleted protein during the early phases of post-irradiation recovery (Slade D and Radman M, 2011). This kind of protein recycling lowers the demand for biosynthesis (Daly MJ *et al*., 2010).

D. radiodurans utilises glucose by glycolysis, gluconeogenesis, and the Pentose phosphate pathway (PPP) (Makarova KS *et al*., 2001). The pentose phosphate pathway (PPP) is highly active in this organism as compared to *E. coli* (Raj HD *et al*., 1960). The PPP process produces substrates which are used in nucleic acid production and as cofactors for antioxidant systems (Slade D and Radman M, 2011).

2.2 *Deinococcus radiodurans*' resistance to DNA damaging agents

D. radiodurans has a high level of resistance to multiple agents that cause different types of DNA damage. The resilience of this bacteria is due to robust antioxidant systems that protect proteins from oxidative degradation and a DNA repairing procedure that accurately reassembles DNA fragments (Daly MJ *et al.,* 2007).

2.2.1 Ionising Radiation

Ionising radiation comes in two forms: particulate (α and β particles) and electromagnetic (X and gamma radiation), both of which are generated by the breakdown of radioactive materials (Cox MM and Battista JR, 2005). Ionising radiation causes approximately 80 kinds of changes in structure, with DNA bases being the most impacted (Slade D and Radman M, 2011). In DNA, gamma rays typically cause 20 SSBs and 1 DSB for every 20 SSBs. A group of radicals can cause strand breaks in both strands at the same location, close-proximity SSBs on opposing strands, or the excision repair of damaged bases that are present on both strands. DSBs inhibit genome replication and cause cell death if they are not repaired (Krisko K and Radman M, 2010).

In *D. radiodurans*, the rate of DSB generation is related to the dose of gamma radiation (Battista JR, 1997). The number of DSBs produced per Gy per genome in radiation-resistant and sensitive organisms is very similar (Gladyshev E and Meselson M, 2008). When *E. coli* and *D. radiodurans* cells were irradiated under the same circumstances, the quantity of DSBs was discovered to be the same in both types of cells (Gerard D *et al*., 2001). In comparison to *E. coli*, which can only reconstruct its genome from 10 to 15 DSB pieces, *D. radiodurans* can repair 1,000 to 2,000 DSBs (Makarova KS *et al*., 2001). Human and yeast cells cannot survive more than 40 DSBs brought on by radiation stress (Rothkamm K and Lobrich M, 2003). These data suggest that proteins and membranes are also key primary targets of radiation damage, in addition to DNA. Through manganese complexes, which are the primary ROS scavengers, proteins defend themselves against oxidative damage (Daly MJ *et al*., 2010).

2.2.2 Desiccation

D. radiodurans have a high level of desiccation resistance. A dry substance with a water content of less than 0.1 g^{-1} H_2O is considered desiccated. Because of protein degradation and ROS production, the loss of water is a frequently fatal event (Potts M, 2001). To shield their cellular macromolecules from desiccation's harmful effects, desiccation-tolerant bacteria, yeast, rotifers use different strategies such as the replacement of lost water molecules by trehalose and sucrose, coating of the DNA molecules by SASPs in *B. subtilis* thus tightly packaging them into a ring-like assembly,

accumulation of the manganese ions and hydrophilins, including LEA proteins (Franca, MB *et al*., 2007).

After six weeks in a desiccation chamber with a five percent humidity content, *D. radiodurans* still has 85% of its viability (Slade D and Radman M, 2011). Around 60 DSBs are produced by these circumstances per genome but it can recreate its genome and restore all metabolic functions upon water delivery (Mattimore V and Battista JR, 1996). High Mn/Fe ratios identify desiccation-resistant bacteria, and these organisms' proteins are less susceptible to oxidation (Fredrickson JK *et al*., 2008). It is anticipated that recently discovered manganese compounds serving as the most effective ROS scavengers will guard proteins against damage brought on by desiccation (Daly MJ *et al.,* 2010).

Trehalose may potentially act as a mediator for protection against desiccation. In the dry state, trehalose forms glasses to replace H_2O lost due to desiccation. This stabilises the dried cytoplasm and lessens the production of free radicals (Slade D and Radman M, 2011). *D. radiodurans* is distinct among bacteria because it has four counterparts of plant desiccation resistant proteins - DR1372, DR1172, DR0105 and DRB0118 (Makarova KS *et al*., 2001). Recently, several additional proteins with intrinsically disorganised hydrophilic regions were discovered; these hydrophilic tails are thought to improve the likelihood that these proteins will remain solvated (Awile O *et al*., 2010).

2.2.3 UV- radiation

Due to the halting of replication forks, exposure to non-ionizing radiation, such as UV rays, slows down the formation of DNA. *D. radiodurans* can successfully restore BPPs and has extraordinary resistance to UV-C radiation (Slade D and Radman M, 2011). Within 90 minutes of exposure to 500 J/m^2 (resistance shoulder) of radiation, *D. radiodurans* cells eliminate more than 80% of the photoproducts produced from thymine (Lai WA *et al*., 2006). BPPs are eliminated by excision repair before DNA replication can continue (Daly MJ *et al*., 2004). Excision and recombination mechanisms are used by *D. radiodurans* to correct the DNA damages. Both the traditional NER pathway (UvrABC) and the endonuclease (UVDE) pathway are available in *D. radiodurans* for the removal of pyrimidine dimers.

Even at high levels, UV does not, in contrast to ionising radiation, cause point mutations in the bacterium (Tanaka M *et al*., 2005). But this bacteria is vulnerable to UV-A (Bauermeister AE *et al*., 2009). UV-C mostly leads to direct DNA damage, whereas UV-A only affects DNA indirectly by generating reactive oxygen species (ROS) (Fernandez Z *et al*., 2006). Oxidative stress caused by UV-A causes irreparable damage to proteins necessary for essential cellular activities. It is possible that *D. radiodurans'* high level of UV-A sensitivity is caused by its sensitivity to oxygen radicals which surpasses its antioxidant systems. Furthermore, it results in lipid peroxidation, which disturbs the integrity of the membrane (Slade D and Radman M, 2011).

2.2.4 Mitomycin C

Interstrand cross-links are produced when the cross-linking agent MMC reacts with guanine (Rajski SR and Williams RM, 1998). MMC produces bis-guanine adducts, which are formed when two guanines are successively alkylated at their N^2 positions. DNA replication and transcription are absolutely blocked by cross-links. *D. radiodurans* have a greater survival rate than *E. coli* and can endure DNA cross-links caused due to this agent. 20g/ml MMC produces 100–200 cross-links per genome in the bacteria. MMC-induced damage requires UvrA and RecA for DNA repair (Slade D and Radman M, 2011).

2.2.5 Base changing chemicals

The bacteria is exceptionally resilient to different base damages, including oxidation (due to ROS, ionising radiation etc), alkylation (produced by ROS and MNNG), as well as deamination (due to MMS and MNNG) (Slade D and Radman M, 2011). *D. radiodurans* is more resilient to hydroxylamine and nitrous acid than *E. coli*. However, it is vulnerable to ethyl methane sulfonate (EMS). It is predicted that *D. radiodurans* would have an effective base excision repair system in operation (Blasius M *et al.,* 2008). **Figure 2** illustrates *D. radiodurans* survivability against gamma rays, desiccation, UV- C radiation, and mitomycin C as compared to *E. coli.*

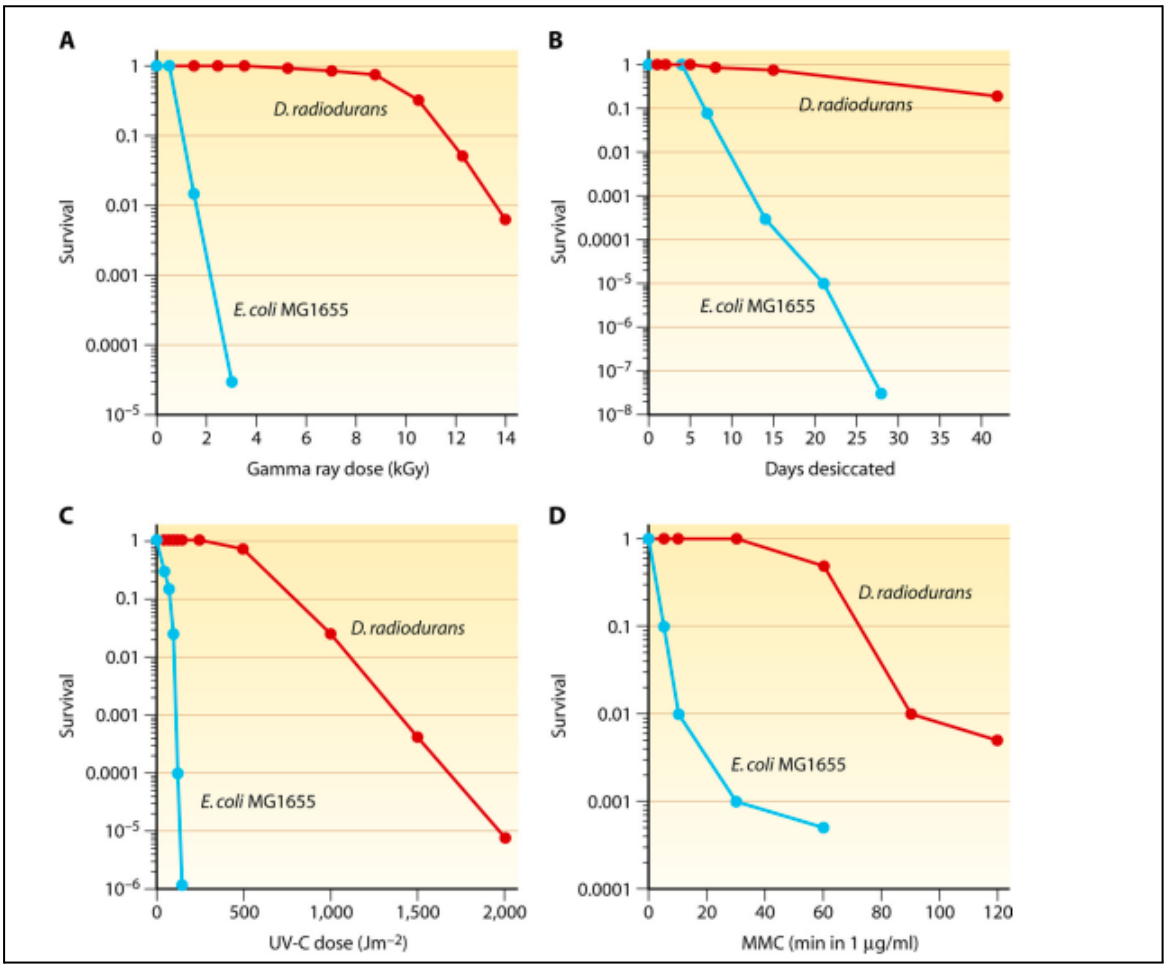


Figure 2: Survival curve *D. radiodurans* survivability against A) gamma rays, B) desiccation, C) UV- C radiation, D) mitomycin C as compared to *E. coli* **(Source**: Slade D and Radman M, 2013**)**

2.3 Factors determining Deinococcal radiation resistance:

The degree of resilience to ionising radiation is greatly influenced by physiological factors, including the culture's age, cell concentration, growth media, pH, irradiation medium, and radiation temperature (Slade D and Radman M, 2011). According to recent studies, cells at late stationary phase are less resilient as compared to those in logarithmic phases (Sukhi SS *et al*., 2009). The high single cell survival rate of *D. radiodurans* suggests that its multicellularity does not increase its radiation resistance (Driedger AA *et al*., 1970) Compared to aqueous solutions, *D. radiodurans* dry cells show greater

radiation resistance, perhaps because water radiolysis and indirect damage are reduced (Slade D and Radman M, 2011).

Cells growing in rich media are more resilient than those developing in minimal media. Irradiated *D. radiodurans* cells need nutrient-rich media for complete recovery. Exposure to radiation at higher pH conditions decreases survival rate, most likely as a result of an increase in oxidative protein damage (Daly MJ *et al*., 2007). Radiation sensitivity is increased when exposed to oxidising chemicals, although decreased when exposed to reducing agents (Duggan DE *et al*., 1963). Cells exposed to radiation at 70°C on dry ice show a better resistance than those at ambient temperature or on ice (Dartnell LR *et al*., 2010).

2.4 Factors contributing to radiation resistance in *D. radiodurans*

The following elements help *D. radiodurans* survive oxidative stress and DNA damage in addition to DNA repair.

2.4.1 Cellular cleansing

In *D. radiodurans*, an antimutagenic system that protects cells from oxidative DNA damage is highly dependent on the transfer and destruction of damaged bases (Slade D and Radman M, 2011). Initially, oligonucleotides that are 2000 base pairs long are produced, together with degraded and undamaged bases (Battista JR, 1997). Nudix hydrolases and nucleotidases detoxify and recycle damaged nucleotides (Xu *et al*., 2001). Damaged proteins must also be degraded in order to be exported from the cell, in addition to damaged DNA. Following exposure to radiation, intracellular proteolytic activity rises (Rouault TA and RD Klausner, 1996). Aconitase, which serves as a sensor for oxidative stress, can activate proteases (Daly MJ *et al*., 2010).

2.4.2 Antioxidant defence

In *Deinococcus radiodurans*, three catalases, four superoxide dismutases, two peroxidases, and two Dps proteins mediate enzymatic ROS scavenging, whereas divalent manganese complexes and carotenoids are nonenzymatic scavengers (Slade D and Radman M, 2011). The defence mechanism of *D. radiodurans* is active against the three

main ROS, including H_2O_2, O_2^-, and OH^-. While dismutases remove O_2^- from the cells, catalases and peroxidases remove H_2O_2 (Tian B *et al*., 2004). Dps proteins attach to DNA, chelate ferrous ions, and convert H_2O_2 to H_2O to protect DNA from oxidative damage (Martinez A and Kolter R, 1997). Deinoxanthin is the main carotenoid in *D. radiodurans*, which remove peroxyl radicals (Tian B *et al*., 2007).

Mn^{2+} complexes are the strongest ROS scavengers in *Deinococcus radiodurans*. The cells have a considerable Mn^{2+} concentration and Mn:Fe ratio (Daly MJ *et al*., 2007). Divalent manganese ions (Mn^{2+}) can remove oxygen from compounds containing phosphate and H_2O_2 from compounds containing bicarbonate, amino acids, or peptides. In addition to being necessary for the action of various endonucleases and superoxide dismutases, Mn also prevents the synthesis of dimers containing thymine (Slade D and Radman M, 2011).

2.4.3 DNA Repair

D. radiodurans has multiple mechanisms to reverse direct DNA damage. DNA synthesis is crucial for DNA restoration in radioactive *D. radiodurans* (Zahradka K *et al*., 2006). The UvrABC pathway uses UvrABC exci-nuclease complex to make cuts at 5’ and 3’ to the damaged spot when alterations in DNA brought on by UV damage is recognized (Petit C and Sancar A, 1999). Cells are protected from DNA degradation brought on by ROS, ionising radiation, and potent alkylating compounds via the BER (UVDE) pathway, which is predominantly powered by a family of uracil DNA glycosylases (Selby CP and Sancar A, 1990). Endonuclease β, which mediates this route, creates a nick 5' to a UV-lesion right away, and requires manganese ions for its activity (Slade D and Radman M, 2011). MutL, MutS1, and UvrD are components of functional mismatch repair mechanism present in *D. radiodurans* (Mennecier SG, 2004).

For the repair of DNA double strand break, several methods were postulated as the primary DNA repair pathways such as SSA, homologous recombination via crossovers, and NHEJ of DNA fragments (Zahradka K *et al*., 2006), out of which SSA and NHEJ have been disproved. For DSBs, two distinct recombinational repair pathways are present (a) ESDSA and (b) homologous recombination involving crossovers that require RecA recombinase (Slade D and Radman M, 2011).

2.4.4 Homologous recombination

In bacteria, this is the primary mechanism used to fix DSBs in the genome. This method creates new DNA lengths by using an entire homologous DNA as a template using DNA polymerase I (Wyman *et al*., 2004). RecBCD protein generates ss 3' DNA overhang and stacks RecA in model bacteria *E. coli*. It performs ATP-dependent nuclease and helicase functions. To prevent further DNA destruction, nuclease activity needs to be strictly regulated. Branch migration can take place prior to the so-called "Holliday junctions" after the DNA ligase has sealed the remaining nicks (Blasius M *et al*., 2008).

RecD protein that is expressed and functional with DNA helicase activity, is produced by this radioresistant bacterium but not RecB or RecC (Makarova *et al*., 2007). A crucial protein needed by strains of *D. radiodurans* for radioresistance is the RecA protein. It is necessary for recombination-based DSBs repair (Gutman *et al*., 1994). According to biochemical analysis of the RecA protein, double-strand DNA is more attractive to RecA than single-strand DNA. RecA has been calculated to have a basal level of approximately 11,000 monomers and a stimulated level of 44,000 monomers in a single cell (Bonacossa de Almeida *et al*., 2002).

2.4.5 Extensive synthesis dependent strand annealing (ESDSA)

The ESDSA model **(figure 3)** proposes that a recessed fragment's single-stranded tail invades a partially overlapping fragment and initiates DNA synthesis via a moving D-loop. Long sections of single-strand DNA are created when the newly synthesised DNA is separated from the template (Blasius M *et al*., 2008). These ssDNA stretches bind to complementary sequences, making it easier to precisely repair long double-strand DNA intermediates. Later, a circular chromosome is created by combining these intermediates once more (Slade D and Radman M, 2011). In the ESDSA model, two enzymes are crucial: PolA, which takes part in the first DNA synthesis stage, and RecA, which guarantees the formation of fully developed chromosomes. Mutations in PolA and RecA are hence very radiation sensitive (Zahradka *et al*., 2006). One aspect of the ESDSA process that is still poorly understood is the priming process during strand extension.

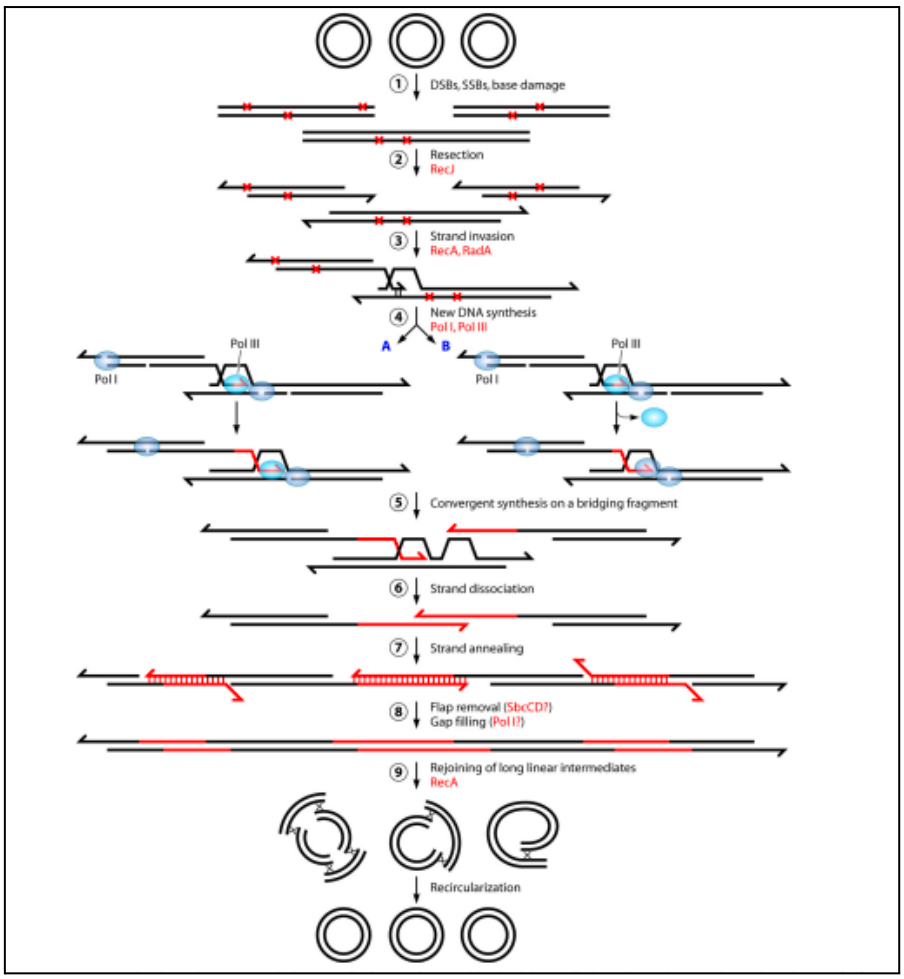


Figure 3: Schematic representation of ESDSA pathway

(Source: Slade D and Radman M, 2011**)**

2.5 DR_1143 gene

The main mechanism of *Deinococcus radiodurans* R1 bacteria to survive high doses of lethal stresses and DNA damage is still not known precisely. Complete analysis of the genome showed that the organism contains many genes that encode proteins with regulatory properties, such as transcription factors, response regulators and kinases (Ying *et al*., 2008). It was discovered that this bacterium responds to stresses by enhancing the expression of many genes. Transcriptomic and proteomic investigations revealed a coordinated activation of genes which support repair of DNA damages, cell cleansing, as well as some hypothesised proteins (Basu B and Apte SK, 2012). Very few genes, such as *recA, uvrA, uvrB, gyrA* and *gyrB* have been found out of the 72 genes upregulated during

first hour following a sub-lethal dose of ionising radiation. In each stress, *ddrA*, *ddrB*, *ddrC*, *ddrD* along with *pprA* genes are most strongly stimulated. Inactivation of these genes displayed radiation sensitive phenotypes which suggest that they are involved in radioresistance (Battista JR *et al*., 2013). However there are some uncharacterized genes present in the genome of *Deinococcus radiodurans* whose function is still unknown, one of which is DR_1143. This gene is upregulated in various stress conditions, indicating its role in radiation resistance of the organism.

DR_1143 NUCLEOTIDE SEQUENCE:

5′ATGACGAAGGACGAATCTCAGGCTTTACAGCAGGCGTTTGCCGAGGTGCGGAAAGAGCAGGCTGCG
GCGCAGGGAAGGTTTCAGAGCCGCGCCGAGGTGCAGCGCACCCAGCGGGACCGCGAGGTGCTGGACC
GGGCGGCGGGGTACACGGTGGACCACATCGTGCGGCAGTCGGCGGACGTGCAGCTCGAAGTCGGCGC
GGTGCTGGCGCAACTGGAGGAGCGGCTTTCGGGCGAGACGTCGAAGCTCGCCGAGCTGGTGACCGCT
GGCGAGGTCGCCGGGCGCGAACTGGCCGAGCTGCGGCGCATCCGGGTGGCCGCCGACGCGCTGGCCG
CCTTGCAGCAAGAAGGCCGTGAACGCGTCGCCCGGCTCCAGACCGAGCATCAGGCGCGGCTGGACGCC
CTGAACCGCGAGCAGAGCGCCGAGCGCCGCGCCTGGGAACGCGAGGACGCCGAGTTCGCCGCAGAAG
AAAGGCGTCAGCAGGAGGAAACGGCCCGTGAGCGCCAGCAGGAGGAAGCCGATCACAGCTACCGGCG
CGAGCGCGAGCGCCAGCACGACGCCGACGCACAGCACGCCGCCGACCGCGCCCAGGAACGCGACCTC
GCCGAACGCCGCCTGACCCTGGAACGCGACTGGCGCGAGCGCGAGGCCGCCCTGCAAGCGGGCGCCG
AGCAGTTCGAGCAAGACCGCATCAAGGTGGAGGCGTTCCCCGCCGAACTCGAAGAAGCCGTCAAAAAG
GCCCGCGAAGAAGGCATCCGGCAGGCCAATGCCGACGCCAAAGTGCGCTCGGACCTGCTGGAACGCG
ACTGGGAAGCGAGCAAGCAGAGCTACGAACTGCACCTCGACTCGCTGCAAGCCGCCGTCGCCGCCGCC
GAAGCGCAGGTCGCCGAGTTGCAGGCCCAGCAGCAGCGCGTCAGCGAACAGACCCAGAACCTCGCCAC
CCGCGCCTTTACCACCGCCAGCACCAGCAGGAGTGAAGCATAA3′

Figure 4: Nucleotide sequence of DR_1143 gene

The coordinated expression of several genes, either grouped in operon(s) in prokaryotes or non-contiguous in eukaryotes, and controlled by a single regulatory mechanism known as a regulon, allows microorganisms to quickly adapt to environmental stimuli (Geisel N, 2011). Trans-acting protein(s) that specifically bind to cis-acting regulatory sequence(s) in the promoter region of the operon(s)/genes it regulates mediate the transcriptional coordination of a regulon (Zhang H *et al*., 2012). Radiation Desiccation Response Motif (RDRM), a 17 bp palindromic cis-regulatory sequence, is crucial for the basal repression and gamma radiation-induced expression of several RDR regulon genes in *D. radiodurans*. These genes consist of 29 ORFs, most of which are arranged non-contiguously, one of which is DR_1143 (Narasimha A *et al*., 2017). The nucleotide

sequence of the gene is 978bp (**figure 4**) while the protein encoded by this gene is 327 amino acids in length (**figure 5)** and its molecular weight is 35kDa.

MTKDESQALQQAFAEVRKEQAAAQGRFQSRAEVQRTQRDREVLDRAAG
YTVDHIVRQSADVQLEVGAVLAQLEERLSGETSKLAELVTAGEVAGREL
AELRRIRVAADALAALQQEGRERVARLQTEHQARLDALNREQSAERRAW
EREDAEFAAEERRQQEETARERQQEEADHSYRRERERQHDADAQHAADR
AQERDLAERRLTLERDWREREAALQAGAEQFEQDRIKVEAFPAELEEAV
KKAREEGIRQANADAKVRSDLLERDWEASKQSYELHLDSLQAAVAAAEA
QVAELQAQQQRVSEQTQNLATRAFTTASTSRSEA

Figure 5: Amino acid sequence of protein encoded by DR_1143 gene

When exposed to gamma radiation, the metalloprotease IrrE (also known as PprI) cleaves the DdrO protein, activating the RDR regulon genes (Wang *et al*., 2015). Sequence conservation, the position of RDRM in relation to the core promoter, and other factors control the degree of activation of several RDR regulon genes. Stressors that cause DNA damage activate the RDR regulon in *D. radiodurans* in a dose and RDRM-/IrrE-dependent manner (Narasimha A and Basu B, 2021).

According to reports, the transcriptional level of this uncharacterized DR_1143 gene is increased several fold during PIR. It has RDRM sequence (TTATGTTtTaAgCgTAA) as well as -10, -35 sequences upstream of the start codon (Narasimha A *et al*., 2017). The order of activation of this gene was found to be gamma rays> MMS> EtBr> desiccation> UV rays> MMC (**figure 6**). The amount of the cellular repressor DdrO is negatively correlated with the degree of activation (Narasimha A and Basu B, 2021).

.

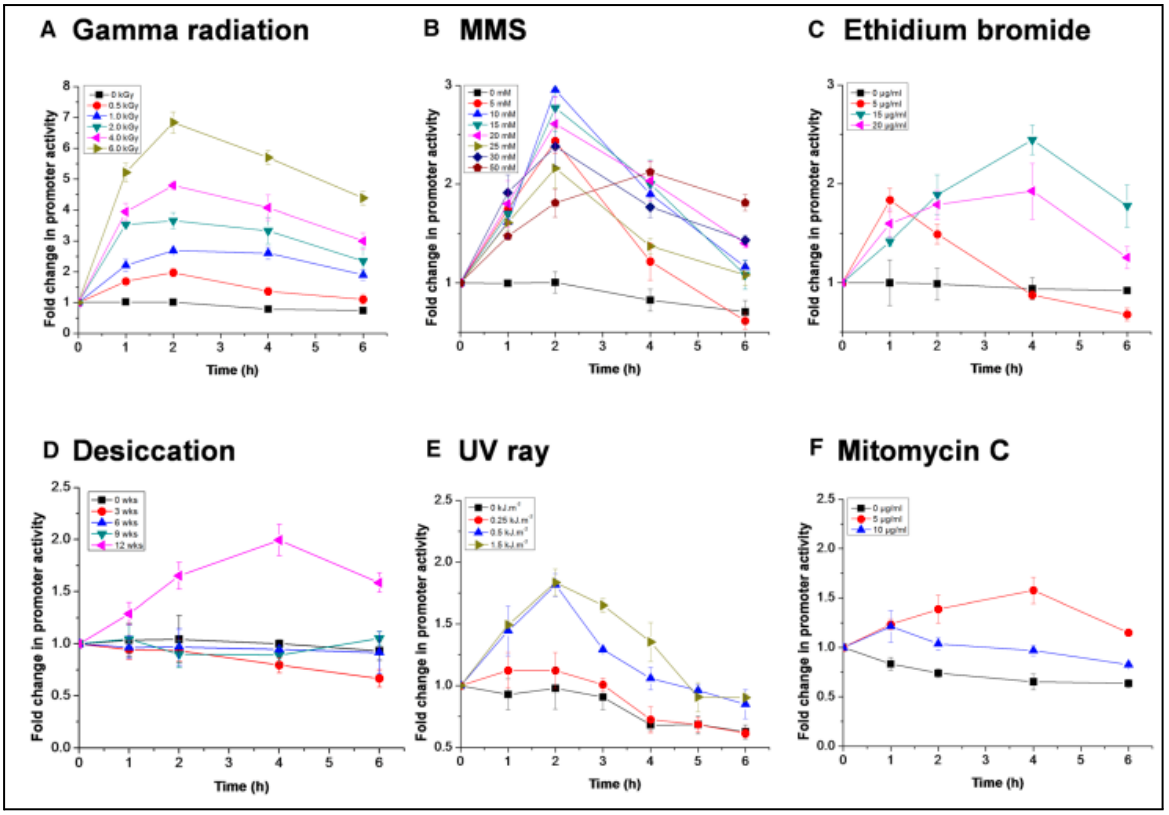


Figure 6: Dynamics of dose-response in P_{DR1143} exposed to various DNA-damaging stimuli **(Source:** Anaganti N and Basu B, 2020**)**

In this study, the DR_1143 gene was successfully amplified from the genome using a gene specific primer set. Following digestion with *EcoRI* and *BamHI* restriction endonucleases, the PCR purified gene was then cloned in pTWIN1 (expression vector) by T4 DNA Ligase. The recombinant vector pTWIN1-DR_1143 was transformed into *E. coli* DH5α cells (cloning strain). After confirmation of cloning by colony PCR, restriction digestion, and Sanger sequencing, the confirmed plasmids were transformed into BL21-Codon Plus RIL cells (*E.coli* overexpression strain). The protein over expression was induced by using IPTG. The whole cell protein was extracted by lysing the cells by sonication. The protein purification was done by affinity chromatography employing chitin beads and Q- Sepharose anion exchange chromatography. DNA- protein interaction studies by EMSA were then carried out which revealed its binding with longer single stranded DNA molecules**.**

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial strains and growth conditions

(a) *Escherichia coli*:

DH5α strain: The *E. coli* strain DH5α was used for cloning purposes. DH5α *E. coli* cells are genetically engineered *E. coli* competent cells having three mutations: recA1, which disables the activity of the recombinases and prevents homologous recombination, endA1, which prevents an intracellular endonuclease from degrading the inserted plasmid, and lacZM15, which allows blue-white screening of transformed cells.

BL21-Codon Plus-RIL strain: BL21-Codon Plus-RIL cells are protease-deficient *E.coli* cells utilised for protein expression research. It contains Lon protease and ompT outer membrane protease deficiencies that limit protein breakdown. They have extra copies of the argU, ileY, and leuW tRNA genes.

E. coli strains used in this study were grown on LB medium at 37°C, maintained under agitation at 150 rpm or on LB agar plates at 37° C. Based on the requirement, carbenicillin (100 µg/ ml) antibiotic was added.

(b) *Deinococcus radiodurans:*

The *Deinococcus radiodurans* R1 (wild type) cell culture was used for genomic DNA isolation. The bacterial cells were grown on TGY media either in broth or on agar plates, kanamycin (8µg/ml) was added wherever required. The cell culture was grown at 32° C in a shaker incubator at 150 rpm. By detecting the O.D. at 600 nm, the growth of both bacterial strains was observed.

3.2 Media preparation

Materials

a) Reagents:

For LB (Luria Bertani) media (100 ml):

(i) 1% Tryptone (1gm), 0.5% yeast extract (0.5 gm) and 1% NaCl (1 gm)

(ii) Agar- 1.7 gm

For TGY (Tryptone, Glucose and Yeast Extract) media (100ml):

(i) 1% Tryptone (1gm), 0.5% yeast extract (0.5 gm) and 0.1% glucose (0.1 gm)

(ii) Agar- 1.7 gm

b) Miscellaneous:

Conical flasks, measuring cylinder, distilled water (Stage-I), beaker, magnetic bead, cotton plug and aluminium foil.

c) Instruments:

Incubators, Laminar Air Flow, Weighing balance, Magnetic stirrer, Autoclave, Oven

Method:

a) To an appropriately sized beaker, add ~80% of the final volume of Stage I distilled water.

b) Add the calculated amounts of above media components. Dissolve the contents with the help of a magnetic stirrer.

c) For the preparation of solid media, add the required amount of agar to the solution.

d) Transfer the solution to Erlenmeyer flasks and close the flask with cotton plug and aluminium foil.

e) Autoclave the media at 121° C and 15 psi.

3.3 Genomic DNA Isolation

Materials

a) Reagents and kits- Genomic DNA Isolation Kit (BRIT**)**

i) Enzymes- RNase A (10mg/ml), Lysozyme (100mg/ml), Proteinase K (20 mg/ml)

ii) Undiluted Buffer PD

iii) Buffer A and B

iv) Buffer C and D- alcohol must be added to these buffers before use as per the requirement based on the number of reactions

v) Absolute alcohol

vi) Nuclease free water/ Buffer E

vii) Columns with collection tubes

b) Miscellaneous: Microfuge tubes (1.5 ml), micropipettes, autoclaved micro tips

c) Instruments: 32° C Shaker Incubator, Centrifuge, Vortex machine

Method

Given below is the stepwise protocol of genomic DNA isolation using Genomic DNA Isolation Kit (BRIT):

a) Inoculate *D. radiodurans* R1 in 5 ml of TGY media.
b) Incubate the culture at 32° C with constant shaking overnight.
c) Transfer it to microfuge tubes. Centrifuge the culture for 1 minute at 13,000 rpm . Load the culture twice or thrice as required.
d) Resuspend pellet in 70% ethanol (700 µl).
e) Mix well by vortexing and then let it stand for 10 minutes by constant mixing.
f) Centrifuge the culture again at 13,000 rpm. 200µl of Buffer A is used to resuspend the pellet .
g) Add 4 µl of Lysozyme, mix well by vortexing for 15 sec. Incubate for 0.5 hour at 37° C.
h) Further, add 4 µl Proteinase K, incubate at 55° C for 30 minutes.
i) Add 2 µl of RNase A and vortex. Incubate at 37° C for five minutes.
j) Add 200 µl of Buffer B, mix well and further incubate for 10 minutes at 65° C.
k) Centrifuge the sample and add 200 µl of absolute alcohol.
l) Load the mixture carefully onto the membrane of the spin column. Centrifuge at 9,000 rpm, 60 seconds.
m) Remove the flow-through and add 500 µl of diluted Buffer C. Spin again at 13,000 rpm.
n) To the column, add 500 µl of diluted Buffer D and again centrifuge at same speed. Eliminate the flow-through and provide an empty spin to get rid of any remaining remnants of Buffer D.
o) Insert the spin column in a microfuge tube. Add 50–100 µl of Buffer E to the column middle, let it rest for 2-3 minutes, and then spin at 13,000 rpm for 60 seconds to elute genomic DNA.
p) The eluted solution may be utilised immediately for further applications or kept at -20° C for additional experimentation.

3.4 Primer design, PCR amplification and purification

3.4.1 Primer Design

a) Data Collection:

The data was collected from databases like KEGG. Genomes, biological pathways, diseases, drugs, and chemical compounds are all covered by the databases that make up KEGG.

b) Method:

1. The DR_1143 gene sequence was downloaded from the KEGG website (https://www.genome.jp/dbget-bin/www_bget?dra:DR_1143).
2. The desired restriction endonuclease sites were checked in the cloning vector (pTWIN1).
3. For forward primer 20-25 bases were taken from the 5' end of DNA sequence of DR_1143. For reverse primer 20-25 bases were taken from the 3' end and reverse complemented.
4. The sequences for appropriate restriction endonuclease sites were incorporated at 5' end of each primer.
5. A few GC bases were introduced at the 5' end of the restriction sites (GC clamp).
6. The primer secondary structure, GC percentage, Tm values and dimer formation among primers were analysed using DNAMAN software (Lynnon Biosoft, Germany). Wherever possible the Tm values of primers were kept around 55 ±5° C and GC content 40-60%.
7. Primers were designed with the forward (DR_1143-F) and reverse primer (DR_1143-R) containing EcoRI and BamHI restriction sites respectively for directional cloning . The DR_1143 gene sequence was then PCR amplified DNA using genomic DNA of *D. radiodurans* as template. The primer sequences are given in **Table 3**. Protocol for preparation of working primer is given in **3.4.1.1**.

3.4.1.1 Preparation of working primer

Materials

a) **Reagents** - lyophilized primers

b) **Miscellaneous -** microfuge tubes, autoclaved micro tips, milli-Q water

c) **Instruments** - centrifuge, vortex machine

Method

a) Spin the vials containing lyophilized oligonucleotides (primers) for 1 minute.

b) Add milli-Q water according to the concentration given by the supplier for achieving 100 pM/µl concentration.

c) For a working primer, the stock solution was diluted into 1:10 ratio by adding milli-Q water to make a working concentration of 10 pM/µl.

d) From this, 0.5 µl of the working primer solution was used for 50 µl of PCR reaction mix.

Bacterial Strains	**Description**	**Source**
DH5α	*E. coli* cloning strain	Lab Stock
BL21-Codon-Plus-RIL	*E.coli* overexpression strain	Lab Stock
D. radiodurans R1	Wild type	Lab Stock
Plasmids	**Description**	**Source**
pTWIN1	Protein expression vector with intein tag, Amp^r	NEB
pTWIN1-g1143	Expression Vector	This study
Primers	**Sequence (5'—3')**	**Restriction Sites used for cloning**
DR_1143-F	GCGAATTCATGACGAAGGACGAATCTCAGC	EcoRI
DR_1143-R	GCGGATCCTTATGCTTCACTCCTGTCGGTGCT	BamHI

Table 3: Bacterial strains, plasmids and Primer sequences

3.4.2 PCR Amplification

Materials

a) Reagent and kit:

i) Q5 kit - Reaction Buffer, GC Enhancer Buffer, dNTPs, High Fidelity Taq polymerase

ii) Primers

iii) DNA Template

b) Miscellaneous:

0.2 ml PCR tubes, micropipettes (2.5, 10, 20, 100, 200, 1000 µl), tips, distilled water (Stage II)

c) Instruments:

Thermocycler: Eppendorf- Master Cycler Nexus Gradient, Centrifuge, Vortex machine

Method:

a) The PCR reaction mixture was prepared as given below:

S.No.	Components	Volume (µl)	Volume (µl)
1.	DNA Template	0.3	0.5
2.	Reaction Buffer (5X)	4	10
3.	GC Enhancer Buffer (5X)	4	10
4.	dNTPs (10 mM)	0.5	2
5.	Forward Primer	0.3	0.5
6.	Reverse Primer	0.3	0.5
7.	High- Fidelity Taq polymerase	0.2	0.5
8.	Milli-Q water	10.4	26
	Total	20	50

b) The PCR amplification programme is given below:

S. No.	Step	Temperature	Time
1.	Initial Denaturation	98° C	30 sec
2.	Denaturation	98° C	10 sec
3.	Primer annealing	55° C	10 sec
4.	Extension	72° C	1 min
Step 2 to 4 repeated for 30 cycles			
5.	Final Extension	72° C	2 mins
Total Time: 1 hour 32 minutes			

c) The PCR amplification was confirmed by loading 5 µl amplified product on 1% agarose gel and electrophoresed at 100 Volts. Protocol for agarose gel electrophoresis is given below in **3.4.2.1**. The positive reactions were identified by comparing with the correct size DNA ladder (1 kb DNA ladder).

d) The amplified PCR product of correct size was PCR purified using PCR purification kit, protocol is given in **3.4.3**.

3.4.2.1 Protocol for Agarose Gel Electrophoresis

Materials

a) Reagents and chemicals

i) Agarose

ii) 1X TBE (Tris borate and EDTA) buffer (composition given in Annexure)

iii) Ethidium bromide (0.5 µg/ml)

iv) Loading dye containing bromophenol blue

b) Miscellaneous: Micropipettes, autoclaved micro tips, milli-Q water

c) Instruments: Weighing machine, microwave oven, agarose gel electrophoresis set up-tray, cassette, combs, lid, and power pack

For visualization of gel: Gel documentation system with CCD camera

For visualization of non-EtBr gel: Blue light transilluminator (Green view)

Method

a) Measure 1 g of agarose into a conical flask, transfer it there, and then add 100 ml of 1X TBE buffer to make 100 ml of 1% agarose solution.
b) Dissolve the agarose by boiling in the microwave until the solution becomes clear.
c) Cool the mixture to about 65° C and add 1-2 µl of ethidium bromide stain.
d) Pour the mixture in a gel casting tray with properly inserted combs in order to form wells.
e) Leave it undisturbed for about 20 min to allow the gel to polymerize.
f) Fill the electrophoresis unit with the 1X TBE buffer after carefully taking off the comb from the gel.
g) To prevent air bubbles from becoming trapped in the solution (gel) tray, insert the gel casting tray within it while tilting it slightly.
h) Place the electrode in unit, cathode is placed towards the well side and anode opposite to the well, sample moves towards the anode.
i) PCR amplified products were mixed properly with appropriate volume of loading dye (composition given in Annexure) and loaded.
j) After electrophoresis, the gel was visualized in a UV transilluminator to observe DNA bands. The image was grabbed using the Gel documentation system with a CCD camera.

3.4.3 PCR product purification

Materials

a) Reagent and Kit: PCR product purification kit (BRIT, JONAKI)

i) Buffer CA

ii) Buffer CB (4 ml alcohol must be added to 1 ml undiluted CB buffer just before use)

iii) Buffer CC/ Nuclease free water

iv) Absolute alcohol

v) Column with collection tubes

b) Miscellaneous: micropipettes, tips, distilled water, microfuge tubes (1.5 ml)

c) Instruments: Centrifuge

Method

a) Add 500 µl CA buffer in PCR sample and stir by a pipette or vortex.
b) Load the mixture very carefully over the spin column that is in the collecting tube. Spin for sixty seconds at 10,000 rpm after sealing the cap.
c) Remove the flow- through and insert the spin column back in the tube.
d) Centrifuge the spin column with 500µl of buffer CB added at 13,000 rpm for 1 minute.
e) Remove the flow-through and empty spin to eliminate remnants of buffer CB.
f) Insert the spin column in a microfuge tube. Add 50 µl of Buffer CC/ milli-Q water to the column middle, let it rest for 2-3 minutes, and then spin at 13,000 rpm for 60 seconds.
g) The eluted solution may be utilised immediately for further applications or kept at -20° C for additional experimentation.

3.5 Restriction Digestion of PCR amplified DR_1143 and pTWIN1 plasmid

Materials

a) Chemicals and reagents:

i) DNA template
ii) CutSmart Buffer(10X)
iii) Stage II distilled water
iv) Restriction Enzymes- EcoRI and BamHI (NEB)

b) Miscellaneous: Micropipette, tips, microfuge tubes

c) Instruments : 37° C incubator

Method

a) The PCR purified DR_1143 and pTWIN1 plasmid **(figure 7)** were digested with restriction endonucleases i.e., EcoRI and BamHI. The reaction mixture was set upas given below and incubated overnight at 37° C.

S. No.	Components	Volume (µl)	Volume (µl)
1.	DNA	1	20
2.	Buffer(10X)	2	5
3.	Enzymes (EcoRI and BamHI)	0.3+ 0.3	1+1
4.	Milli- Q water	16.4	23
5.	**Total**	20	50

b) After incubation, 5 µl of the digested DNA was electrophoresed in 1% agarose gel using DNA electrophoresis protocol given in **3.4.2.1** to check if complete digestion took place.

c) This digested DNA was then loaded onto non- ethidium bromide gel and the correct size DNA fragment was eluted from gel and used for ligation. The protocol for agarose gel extraction is given below in **3.5.1.**

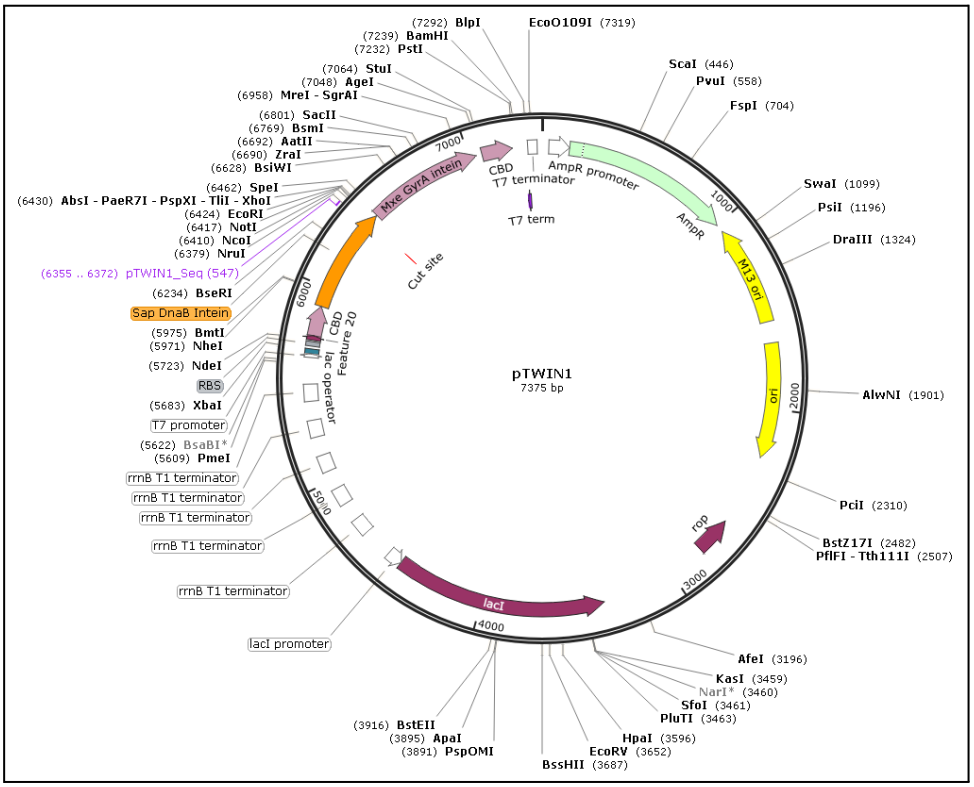


Figure 7: Schematic representation of pTWIN1 vector

(Source: https://www.snapgene.com**)**

3.5.1 Protocol for Agarose gel extraction

Material

a) **Reagent and Kit:** Gel Extraction Kit- Monarch NEB (New England Biolabs)

i) Dissolving buffer

ii) DNA wash buffer- diluted with alcohol

iii) Elution buffer

iv) Column with collection tubes

v) Novel Juice DNA stain (6X)

b) Miscellaneous: Micropipettes, milli-Q water, microfuge tubes (1.5 ml)

c) Instruments: Centrifuge

Method

a) Add 5 µl of Novel Juice dye to PCR product/ restriction digestion DNA sample and load on wells. Electrophoresis is carried out at 50 V.
b) Use a sharp scalpel to separate the agarose gel bearing the desired DNA band, then put the gel in a microfuge tube.
c) Put 1000 µl of Gel Dissolving buffer into the microfuge tube containing the gel piece with the DNA of interest.
d) To fully dissolve the gel, incubate it at 50–52 °C for 5 minutes. Mix the contents by flipping the tube every 2-3 minutes while the gel is being incubated to aid in the gel's dissolution.
e) Transfer the mixture to the spin column and centrifuge at 13,000 rpm for 1 minute. load twice or thrice as required.
f) Remove flow-through and put 200 µl of Wash buffer-I in the column. Spin again in the same conditions. Eliminate the flow-through.
g) Add 200 µl of Wash buffer-II and centrifuge again at 13,000 rpm.
h) Remove the flow-through and empty spin briefly to remove remnants of ethanol.
i) Insert the spin column in a microfuge tube. Put 20µl of Milli- Q water to the column middle, let it rest for 2-3 minutes, and then spin at 13,000 rpm for 60 seconds to elute the sample.

3.6 Ligation of DR_1143 into pTWIN1 plasmid

Materials

a) Chemical and reagents: Sigma Quick Ligation Kit

i) Ligase enzyme from T4 infected E.coli

ii) Ligation buffer (5X)

iii) ATP

b) Miscellaneous: micropipettes, 0.5 ml microfuge tubes

c) Instruments: Centrifuge

Method

The digested DNA fragment of DR_1143 was ligated into pTWIN1 plasmid using a ligation kit (Sigma Quick Ligation Kit). The reaction mixture was kept for a minimum 3 hours at room temperature.

S. No.	Components	Volume (µl)
1.	Template DNA (plasmid)	4 µl
2.	Insert DNA	4 µl
3.	Ligation Buffer	10 µl
4.	ATP	1 µl
5.	T4 ligase enzyme	1 µl
	Total	20 µl

3.7 Transformation of pTWIN1-DR_1143 genetically modified vector into competent *E. coli* DH5α cells

a) The pTWIN1- DR_1143 genetically modified vector was transformed into DH5α cells. Protocol for *E. coli* competent cells and its glycerol stock preparation is given in **3.7.1** and **3.7.2** respectively. The transformation protocol is discussed in **3.7.3.**

b) The transformed cells were spread on LB agar plates with appropriate antibiotic and incubated at 37° C overnight.

c) Colonies were randomly selected and patched again on fresh plates.

d) Colonies were picked up to confirm the presence of the insert DNA fragment in the vector by colony PCR. Protocol for colony PCR is given below in **3.7.4**.
e) Second confirmation was done by restriction digestion to "insert out" the DNA fragment. The protocol for restriction digestion is discussed in **3.5.**
f) Finally, the cloning was confirmed by Sanger Sequencing.
g) The positively transformed colonies were selected for plasmid isolation using plasmid isolation kit (BRIT). Protocol for plasmid isolation is given below in **3.7.5**.
h) Isolated plasmids were used further for transformation into BL21-Codon Plus-RIL cells.

3.7.1 Competent cell preparation protocol for *E.coli*

Competent *E. coli* cells of various strains were made by calcium chloride-rubidium chloride method.

Materials

a) **Reagents:** LB media, antibiotic, solution A and B (Composition given in Annexure), glycerol, DH5α Host cells stock
b) **Instruments:** Centrifuge, Laminar Air Flow, 37° C incubator shaker
c) **Miscellaneous:** Centrifuge tubes, ice, microfuge tubes, micropipettes, autoclaved micro tips, sterile eppendorf tubes

Method

a) Inoculate a colony of DH5α strain in 5 ml LB media containing carbenicillin antibiotic.
b) Incubate in the 37° C shaker overnight.
c) Reinoculate 100 µl of the culture in fresh LB media and incubate at 37° C in the shaker till OD_{600} reaches 0.5.
d) Transfer the culture in the centrifuge tube, then spin it at 5,000 rpm at 4 °C for 5 minutes.
e) Remove the supernatant and resuspend the pellet in 5 ml of solution A (aseptically), mix it gently, and centrifuge it at 5,000 rpm for 5 minutes at 4° C.
f) Resuspend the pellet in 5 ml of solution B (aseptically), discard the supernatant, gently stir, and place on ice for 30 minutes.

g) Centrifuge it at 5,000 rpm for 5 minutes at 4° C.

h) Add 1 ml of Solution B (aseptically) to the pellet , mix it properly.

i) Aliquot 150 µl of this solution in microfuge tubes. Store at -70° C with 15% glycerol. The protocol for glycerol stock preparation is given below in **3.7.2.**

3.7.2 Glycerol Stock Preparation

Materials

a) Reagents and chemicals: sterile glycerol solution, liquid N_2 (-196° C)

b) Miscellaneous: sterile microfuge tubes (1.5 ml), micropipettes, autoclaved micro tips, stage II distilled water

c) Instruments: vortex machine, laminar flow hood

Method

a) Under sterile conditions, 1 ml culture was added to 250 µl glycerol solution (final concentration -20%).

b) The suspension was vortexed and labelled properly.

c) This suspension was then stored at -196° C in liquid N_2.

d) An aliquot was streaked on agar plates with a nichrome inoculation loop in order to revive the culture from frozen stock, and it was then cultured at the proper temperature with the right antibiotic for the specified amount of time. One colony from each of these plates was inoculated in the liquid broth, allowed to grow overnight, and then used in the various experiments.

3.7.3 Transformation protocol for *E. coli*

Materials

a) Requirements: LB agar and broth media, DH5α competent cells**,** plasmid DNA/ ligation mix**,** antibiotic- carbenicillin

b) Instruments: Laminar Air Flow, 37° C shaker incubator, centrifuge, 37° C plate incubator

c) Miscellaneous: Water bath, ice, spreader, 70% ethanol, micropipettes, autoclaved micro tips

Method

a) In 150 µl of competent cells, 20 µl ligation product/ 0.5 µl of plasmid was added and mixed by tapping. The mixture was kept on ice for 1 hour.
b) Heat shock was given for 90 seconds at 42° C and then kept for 5 minutes on ice.
c) 1 ml of sterile LB media was then added to it and incubated in a 37°C shaker incubator for recovery for 1 hour .
d) The transformation mix was spun at 6,000 rpm, 3 minutes.
e) More than half of the supernatant was discarded, leaving approximately 200 µl of it. The pellet was resuspended in the remaining supernatant and spread on LB agar plate containing carbenicillin antibiotic.
f) The plates were incubated at 37° C overnight.

3.7.4 Colony PCR protocol

Materials

a) Reagents and kit:

i) PCR Mastermix – Renwik (Store at -20° C): Buffer, dNTPs, Taq polymerase

ii) Primers

iii) DNA Template

b) Miscellaneous: Micropipette, Stage I distilled water, microfuge tubes

c) Instruments: Thermocycler, Agarose gel electrophoresis set up

Method

a) The reaction mixture for colony PCR was prepared as follows:

S. No.	Components	Volume (µl)
1.	Buffer (10X)	2
2.	dNTPs	0.5
3.	Forward Primer	0.3
4.	Reverse Primer	0.3
5.	Taq polymerase	0.3
6.	Milli-Q water	16.1
7.	Template	-
	Total	20

b) Dispense 20 µl of PCR master mix into PCR tubes.

c) Transfer the cells from each colony to a PCR mastermix using a sterile micropipette tip. Stir briefly to resuspend the cells in the master mix.

d) Amplification was carried out using the programme given below

S. No.	Step	Temperature	Time
1.	Initial denaturation	95	3 minute
2.	Denaturation	94	15 second
3.	Annealing	55	15 second
4.	Extension	72	1 minute
Step 2 to 4 repeated for 30 cycles			
5.	Final extension	72	5 minutes
Total Time: 1 hour 32 minutes			

e) The analysis of PCR products is carried out by performing Agarose Gel Electrophoresis.

3.7.5 Protocol for Plasmid Isolation

Materials

a) Reagents and kits: Monarch Plasmid Miniprep Kit- NEB

i) Wash buffer I

ii) Wash buffer II

iii) Resuspension Buffer (B1)

iv) Lysis Buffer (B2)

v) Neutralization Buffer (B3)

vi) Elution buffer

vii) Column with collection tubes

b) Miscellaneous: microfuge tubes

c) Instruments: Centrifuge

Method:

a) Transfer the single bacterial colony from the positive colonies in 5 ml LB media containing carbenicillin antibiotic. Keep the culture in a 37°C shaker incubator overnight.
b) Centrifuge 3 ml culture at 13,500 rpm for 1 minute. Load the culture twice or thrice as required.
c) Add 200 µl Resuspension Buffer (B1) to the pellet, mix by pipetting or vortex.
d) Add 200 µl Lysis Buffer (B2) and gently mix . 1 minute of incubation at room temperature is required.
e) 200 µl Neutralisation Buffer (B3) should be added and gently mixed until the colour is evenly yellow and a precipitate forms. 2 minutes of room temperature incubation is required.
f) Centrifuge for 6 minutes at 13,500 rpm to get a thick pellet.
g) Transfer the supernatant onto a spin column and centrifuge at 13,000 rpm for 1 minute.
h) Eliminate flow-through, add 200 µl Wash Buffer I and spin at 13,500 rpm for 1 minute. Add 200 µl of Plasmid Wash Buffer II and centrifuge again. Empty spin to remove traces of ethanol.

i) Add 50-60 µl of Elution buffer/ Stage II distilled water and spin at 13,500 rpm for 1 minute to elute the DNA.

3.8 In-silico analysis

3.8.1 Similar sequence search

Tool used

The BLAST (Basic Local alignment Search Tool) identifies areas where sequences are locally similar. The tool evaluates the likelihood of matches between nucleotide or protein sequences.

Method

a) On the NCBI server, click on BLASTp and enter the amino acid sequence in FASTA format.

b) In parameters, exclude Deinococcus radiodurans (taxid: 1299).

c) Select 'BLOSUM45' as matrix and run BLAST **(figure 8)**.

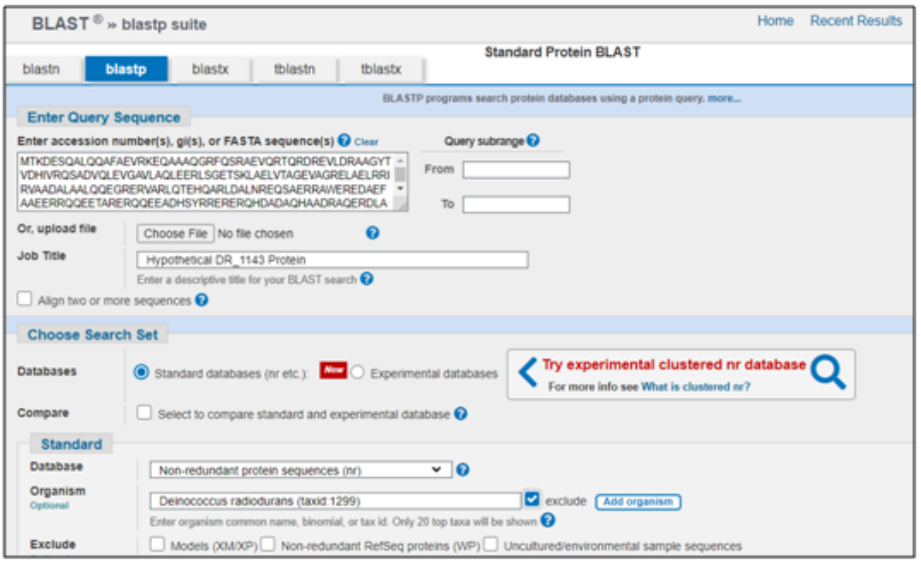


Figure 8: Blastp (Protein BLAST) parameters for identifying sequences similar to DR_1143 protein sequence

3.8.2 Tertiary structure prediction

Tool used:

Phyre2 is one of the most widely used techniques for predicting protein structure based on structural similarity.It uses the concept of homology modelling for predicting the 3-D structure of proteins.

Method:

a) On Phyre [2] server, enter email id and amino acid sequence.

b) Click on Phyre search.

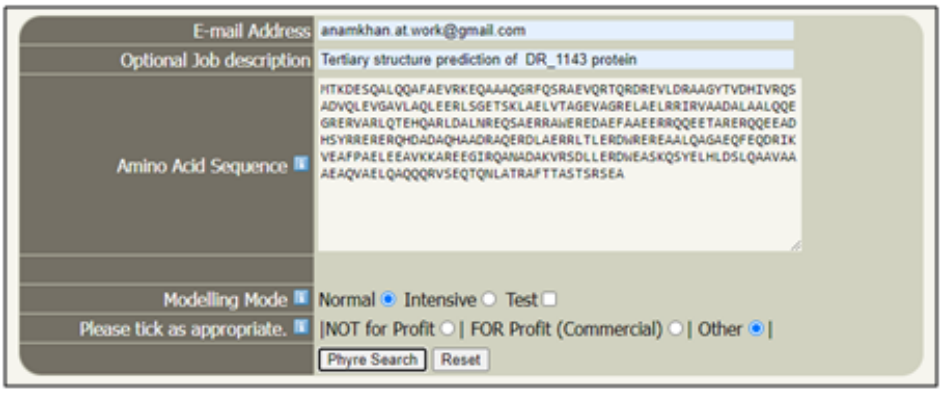


Figure 9: Phyre[2] parameters for tertiary structure prediction of DR_1143 protein sequence

3.9 Over-expression, Extraction and Purification of DR_1143 protein

a) After confirming the sequence of cloned gene by digesting with EcoRI and BamHI pTWIN1- DR_1143 and pTWIN1 (negative control) were transformed into competent *E.coli* BL21-Codon Plus- RIL cells which is an overexpression host strain.

b) The competent cell preparation protocol for *E.coli* BL21-Codon Plus- RIL cells is the same as for DH5α cells given in **3.7.1**.

c) Transformation protocol was the same as in **3.7.3**.

d) The transformed *E.coli* BL21-Codon Plus- RIL cells were spread on LB plates with antibiotics and kept in a 37° C shaker incubator overnight.

e) Colonies were randomly selected and patched on fresh plates. DR_1143 protein over-expression was induced by addition of IPTG (0.5 mM). Protocol for protein over-expression and extraction is discussed in **3.9.1.**

f) The protein extract was resolved using 12% SDS-PAGE gel, the protocol is given in **3.9.2**.

g) The extracted protein was purified by Affinity and Ion exchange chromatography; the protocols are discussed in **3.10.**

3.9.1 Over-expression of DR_1143 protein and extraction

Materials

a) Reagents and chemicals:

i) LB broth and agar media

ii) Carbenicillin antibiotic

iii) IPTG (0.5 mM)

iv) 1X PBS

v) 20 mM Tris Buffer (pH 8.0)

b) Miscellaneous: microfuge tube, micropipettes, autoclaved microtips, stage II distilled water, ice

c) Instruments: 37° C shaker incubator, centrifuge, sonicator- Branson Digital Sonifier

Method

a) A colony from transformed plates was inoculated in LB medium with carbenicillin antibiotic and incubated at 37° C till $O.D_{600}$ reaches 0.5. The starter culture was reinoculated in a 1:100 dilution ratio.

b) IPTG (0.5 mM) was added to the culture and incubated at 20° C in a shaker overnight.

c) The culture was centrifuged at 5,000 rpm for 10 minutes at 4° C.

d) The pellet was resuspended in 1X PBS and centrifuged at 10,000 rpm for 6 minutes.

e) 20mM Tris Buffer (pH 8.0) was added to the pellet and sonication was carried out for cell lysis (Total time- 5 minutes, 2 seconds on, 2 seconds off, amplitude- 30%).

f) The sonicated extract should be kept on ice.

g) The sample was centrifuged at 30,000 rcf for 30 minutes (4° C) to remove cell debris.

h) 10 µl of the supernatant was mixed with 2 µl of 5X SDS Cracking Buffer and loaded on 12% SDS-PAGE gel as discussed in 3.8.2.

3.9.2 SDS-PAGE Gel electrophoresis

Materials

a) Reagents and chemicals:

i) 30% Acrylamide- bisacrylamide solution

ii) 0.5M Tris (pH 6.8)

iii) 10% SDS

iv) 10% APS

v) TEMED

vi) 0.5M Tris (pH 8.8)

vii) Acetone

viii) Isopropanol

ix)1X Tris-glycine- SDS Running buffer

x) Coomassie Brilliant Blue (CBB-G-250) dye and destaining solution (Compositions given in Annexure)

b) Miscellaneous: micropipettes, autoclaved micro tips

c) Instruments: Protein electrophoresis apparatus, power pack, gel rocker

Method

a) **Preparation of 12% separating gel**- The components were added as follows-

Resolving get - 12% (20 ml)		
S. No.	**Reagents**	**Volume (ml)**
1.	Water	6.59
2.	Acryl-Bisacryl	8
3.	1.5M Tris (pH8.8)	5
4.	10% SDS	0.2
5.	10% APS	0.2
6.	TEMED (added at last)	0.01

The gel was poured between plates and a small amount of isopropanol was poured above the gel to remove the air bubbles. It was allowed to polymerize for an hour. After polymerization, isopropanol was removed with distilled water.

b) **Preparation of 5% Stacking gel**- Following components were added -

Stacking gel 5% (10 ml)		
S. No.	**Components**	**Volume (ml)**
1.	Water	5.622
2.	Acryl- Bisacryl (30%)	1.667
3.	1.5M Tris (pH6.8)	2.5
4.	10% SDS	0.1
5.	10% APS	0.1
6.	TEMED (added at last)	0.01

The stacking gel mix was applied on top of the resolving gel and a comb was attached to form wells for sample loading. It was allowed to polymerize for an hour.

c) The unit was filled with the 1X Tris-Glycine-SDS Gel Running Buffer and the combs were then removed.

d) Sample Preparation- Protein samples for loading onto SDS-PAGE gel were prepared by mixing 40 µl of the cell-free extract solution and 10 µl of 5X SDS Cracking buffer, mixed by vortexing.

e) The mixture, after a short spin, was loaded onto the gel (about 10 µl). Electrophoresis was carried at 50V till the dye front crossed the stacking gel and then increased to 100V.

f) The gel was removed from the glass cassette, rinsed with water and stained for about 2 hours in CBB and then destained. The solution was changed till the gel background became clear. The gel image was recorded with the Gel documentation system (SYNGENE).

3.10 Protein purification

3.10.1 Affinity Chromatography

The chitin-binding domain(s) in the intein-tag(s) enables a fusion protein to be affinity purified. Due to the CBD's strong affinity for chitin beads, the protein can be effectively recovered using the following procedure, which uses chitin resin (NEB#S6651):

Materials:

a) Reagents and chemicals:

i) Matrix- Chitin resin (NEB#S6651)

ii) Column buffer (20 mM Tris-HCl, pH 8.5, 0.5 M NaCl)

iii) Cleavage Buffer (20mM sodium phosphate buffer, pH 6.0, 0.5 M NaCl)

iv) 0.3 M NaOH

v) 20mM Tris- HCl, pH 8.0 and 25 mM NaCl buffer

b) Miscellaneous: chromatographic column, stage II distilled water, falcon tubes, dialyzing membranes, beaker, micropipettes, autoclaved microtips, 70% ethanol

c) Instruments: hot plate, magnetic stirrer

Method

a) **Chitin Column Preparation**- A column was packed with 20 mL of chitin bead slurry to purify 1 L of culture and equilibrated with column buffer (20 mM Tris-HCl, pH 8.5, 0.5 M NaCl).

b) **Sample loading**- The sample was loaded on the chitin column slowly and flow through (FT) was collected.

c) **Column washing** - The chitin resin was washed 2-3 times with the column buffer and wash (W) was collected.

d) **On-column cleavage induction**- Washing the column with Cleavage Buffer (20 mM sodium phosphate buffer, pH 6.0, 0.5 M NaCl) caused the on-column cleavage to occur. Upon saturation of the column with the phosphate buffer, it was incubated at 4° C overnight.

e) **Elution of the target protein**- The target protein was eluted after washing with the cleavage buffer. 2-2 ml fractions were obtained and subjected to 12% SDS-PAGE

analysis. The desired protein was released, however the intein-CBD tag remained attached to the resin.

f) **Stripping and regeneration of resin**- The uncleaved proteins and intein-tag were removed from the chitin resin using 0.3M NaOH . Before stripping, a tiny portion of the resin was kept, and the resin was then washed with the stripping solution. It was left to incubate in the stripping solution for 30 minutes, and then washed over with it again. The column was then washed with water. The regenerated resin was kept at 4 ° C.

g) **Dialysis of the target protein**- The dialyzing tubes were rinsed in distilled water with vigorous boiling to remove the glycerol coating and autoclaved. A clamp was secured to one end of the membrane and the eluted fractions were then transferred to the membrane from the other end which was then clamped. The membranes were immersed in an appropriate buffer (20mM Tris- HCl, pH 8.0 and 25 mM NaCl) and kept at 4° C overnight.

h) The eluted fractions were then passed over a Q-Sepharose column and eluted by a NaCl (25 mM to 500 mM) gradient in order to eliminate minute quantities of contaminants, protocol for anion exchange chromatography is discussed below in **3.10.2.**

3.10.2 Q-Sepharose Anion Exchange Chromatography

Q Sepharose Fast Flow is an ion exchange chromatographic resin with a quaternary amine (Q) functional group attached [-CH2-N + $(CH3)_3$]. The Q group is a powerful anion exchanger that is totally ionised for a broad pH range. The protocol is discussed below-

Materials

a) Reagents and chemicals:

i) Resin- Q-Sepharose Fast Flow

ii) Start Buffer (20mM Tris- HCl, pH 8.0 and 25 mM NaCl)

iii) Elution Buffer (20mM Tris- HCl, pH 8.0 and 500 mM NaCl)

iv) Storage buffer- 20mM tris (pH 8.3), 500 mM NaCl, 1 mM EDTA, 1 mM β-Me, 50% glycerol

b) Miscellaneous: chromatographic column, stage II distilled water, falcon tubes, dialyzing membranes, beaker, micropipettes, autoclaved microtips, 70% ethanol

c) Instruments: hot plate, magnetic stirrer

Method

a) The column was placed vertically on a suitable platform, away from direct sunlight or drafts that could induce temperature variations. The column was washed with 70% alcohol and water.

b) **Equilibration-** The column was loaded with the resin slurry and the start buffer (20mM Tris- HCl, pH 8.0 and 25 mM NaCl) was used to equilibrate the column.

c) **Loading-** The sample dissolved in the start buffer was applied to it and flow through was collected.

d) **Washing-** Wash was collected after washing the column with the start buffer.

e) **Elution-** Elution was carried out with NaCl solution of rising ionic strength from 20 mM to 500 mM. 3-3 ml fractions were collected, which were assessed by 12% SDS-PAGE.

f) **Dialysis-** Finally, the eluted fractions were dialysed using storage buffer – [20mM tris (pH 8.3), 500 mM NaCl, 1 mM EDTA, 1 mM β-Me, 50% glycerol] and kept at 4° C overnight.

3.11 Protein estimation by Folin Lowry Method

The Lowry method is based on the reduction of Cu^{+2} to Cu^{+}, produced by the oxidation of peptide bonds under alkaline conditions (the Biuret test), and the subsequent reduction of the Folin Ciocalteu reagent to heteropoly molybdenum blue by the copper-peptide bond complex with the oxidation of aromatic amino acids.

Materials

a) Reagents and kit- Sigma Total Protein Kit, Peterson's Modification

i) Lowry reagent, powder

ii) 0.15% Deoxycholate (DOC) solution

iii) Trichloroacetic acid (TCA) solution

iv) Folin and Ciocalteau's phenol reagent

Standard solution- BSA (1 mg/ml)

b) Miscellaneous: Microfuge tubes (1.5 ml), micropipettes, autoclaved micro tips, autoclaved distilled water

c) Instruments: Centrifuge, vortex machine, water bath

Method

a) 500 µl of water was added to a microcentrifuge tube with the label "Blank".

b) The protein standards (S1, S2, S3, S4, S5) and the samples (F1, F2, F3, F4, F5) were added to the appropriately labelled tubes and diluted with water to 500 µl.

c) 50 µl of the DOC Solution and 50 µl of the TCA Solution were then added, vortexed and incubated for 10 minutes at RT.

d) The mixtures were spun down for 10 minutes at 13,000 rpm to pellet down the precipitate. Each pellet received 500 µl of water, which was vortexed into a thorough mixture.

e) The Lowry Reagent was made by dissolving the powder in 40 ml of water. Each tube received 500 µl of the Lowry Reagent, which was then vortexed and left at room temperature for 10 minutes.

f) 250 µl of the Folin's Reagent was further applied, vortexed and incubated in dark for half an hour.

Tabulation

S. No.	Sample name	Vol. of sample (µl)	Vol. of distilled water (µl)	Conc. of sample (µg/µl)	Vol. of DOC solution (µl)	Vol. of TCA solution (µl)	Vol. of Lowry Reagent (µl)	Vol. of Folin Reagent (µl)
1.	Blank	0	500	0	50	50	500	250
2.	S1	2		2				
3.	S2	4		4				
4.	S3	6		6				
5.	S4	8		8				
6.	S5	10		10				
7.	F1	1		?				
8.	F2	1		?				
9.	F3	1		?				
10.	F4	1		?				
11.	F5	1		?				

g) The absorbance was measured spectrophotometrically at 760 nm using the Magellan software program.

3.12 Determination of oligomeric status of purified proteins

The oligomeric state of purified proteins was determined by glutaraldehyde cross-linking.

Materials

a) Reagents and chemicals

i) 2.5% glutaraldehyde solution

ii) 50 Mm sodium phosphate buffer (pH 8.0)

iii) 1 M Tris (pH 8.5)

b) Miscellaneous: micropipettes, autoclaved micro tips

c) Instruments: 37° C incubator, protein electrophoresis apparatus, power pack, gel rocker

Method

a) 25 µg of individual protein were combined with 5 µl of freshly diluted 2.5% glutaraldehyde in a 50 µl reaction mix having 50 mM sodium phosphate buffer (pH 8).

b) The mixture was allowed to incubate for 5 minutes at 37°C.

c) The reaction was stopped through the addition of 10 µl 1 M Tris-HCl (pH 8.5), and then resolved using 12% SDS-PAGE, stained with CBB-G-250 dye and finally destained.

3.13 Native Page for DNA: Protein complexes

Native PAGE analysis is a crucial method for resolving DNA-protein interactions in their native condition.

Materials

a) Reagents and chemicals:

i) 30% Acrylamide- bisacrylamide solution

ii) 40 mM Tris (pH 7.4)

iii) 10% APS

iv) TEMED

b) Miscellaneous: micropipettes, autoclaved micro tips

c) Instruments: Protein electrophoresis apparatus, power pack, gel rocker

Method

a) The native- PAGE gel was prepared as follows-

Native Page gel (6%)			
S. No.	**Reagents**	**Volume (ml)**	**Volume (ml)**
1.	Water	15	22.25
2.	Acryl-Bisacryl (30%)	4	0.3
3.	40 mM Tris (pH7.4)	0.8	1.2
5.	10% APS	0.2	0.3
6.	TEMED (added at last)	0.015	0.0225
	Total	20	30

b) The gel solution was then applied between two 1 mm thick glass plates (10 x 8 cm). Using combs of standardised sizes, appropriate wells were created as the solution was being poured.

c) The gel was left to polymerize and used for resolving DNA-protein complexes.

3.14 DNA labelling with a non-radioactive DIG label

Materials

a) Reagents and chemicals

i) Digoxigenin labelled ddUTP

ii) Terminal transferase enzyme

iii) Labelling buffer

iv) $CoCl_2$

v) EDTA (0.2 M)

b) Miscellaneous: Superdex 50 columns, micropipettes, autoclaved micro tips, microfuge tubes, molecular biology grade water

c) Instruments: 37° C incubator, centrifuge, Thermocycler

Method

a) The DNA oligos were labelled with Digoxigenin labelled ddUTP using terminal transferase enzyme. The reaction mixture was prepared as follows:

S. No.	Components	Volume (µl)
1.	DNA oligo	2
2.	Molecular Biology Grade Water	18
3.	Labelling Buffer	8
4.	$CoCl_2$	8
5.	ddUTP	2
6.	Terminal transferase enzyme	2
Incubated at 37° C for 30-40 minutes		
7.	EDTA (0.2 M)	4
8.	Molecular Biology Grade Water	6
	Total	50

b) The reaction was halted by adding EDTA (final concentration of 0.02 M).

c) The labelled oligos were purified using mini superdex 50 columns on the basis of size exclusion chromatography. An empty spin was given to the superdex columns followed by two washes with water at 1000g for 1 minute. The columns were inserted in microfuge tubes and the reaction mixture was added. The columns were centrifuged at 1000g for 3 minutes and the purified labelled DNA was finally eluted.

d) Primer annealing was carried out by heating the PCR tubes containing the oligonucleotides to 95° C for 2 minutes (initial denaturation) and then allowing the temperature to 10° C slowly.

3.15 Electrophoretic Mobility Shift Assay (EMSA)

The Electrophoretic Mobility Shift Assay (band shift assay or gel retardation assay) is used to study how proteins interact with nucleic acids. The foundation of EMSA is the idea that DNA and protein complexes travel more slowly over nondenaturing

polyacrylamide gel than single DNA fragments or double-stranded oligonucleotides. The following DNA samples were used in the assay:

3.15.1 Oligo DT-50 and M-13 DIG Labelled DNA

Materials

a) Reagents and chemicals-

i) 20 mM Tris-acetate pH 7.4

ii) 1 mM $MnCl_2$

iii) 10X native dye

iv) 0.5X TBE buffer

v) Blocking solution [1% of blocking reagent (Roche, UK) in 100 mM maleic acid, 150 mM NaCl buffer, pH 7.5]

vi) 40 mM Tris acetate, pH 7.4

vii) TS buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl)

viii) Anti- DIG antibody coupled to alkaline phosphatase

ix) NBT/BCIP

b) Miscellaneous: micropipettes, autoclaved micro tips, distilled water

c) Instruments: Electrophoresis unit

Method

a) The reaction mixture for EMSA studies with oligo DT-50-DIG (a homopolymer) and M-13-DIG labelled DNA (a heteropolymer) was prepared as follows and left to incubate for 60 min at room temperature -

S. No.	Components	Volume (µl)
1.	$MnCl_2$ (10 mM)	2 µl
2.	Tris Acetate (20 mM) pH 7.4	2 µl
3.	DNA	1 µl
4.	Protein (1 µM)	0-3 µl
5.	H_20	Rest of the volume
	Total	20 µl

b) 2 µl of 10X native dye was added to the reaction mixture and the DNA-protein complexes were separated using 6% native PAGE for 3 hours at 50 volts in a buffer solution of 40 mM tris acetate, pH 7.4.

c) **Electroblotting of DNA:Protein Complexes-** Proteins that had been resolved by SDS-PAGE were electro blotted on a nylon membrane. The blotting process was carried out in TBE buffer for 0.5 hours at 400 milliamps. Air bubbles were carefully avoided. DNA-protein complexes were cross-linked by exposure to UV light and the membrane was incubated at 4° C overnight.

d) **Immunodetection of DNA:Protein complexes:** The membrane was then placed in 10 ml blocking solution for 10-15 minutes. The complexes were then probed with an anti-DIG antibody in blocking solution (Dilution 1:5000) for 2 hours. Following three rounds of washing in the TS buffer, NBT/BCIP was used to develop the membrane.

3.15.2 Single and double stranded DNA of ΦX174 Virion

Materials

a) Reagents and chemicals:

i) 20 mM Tris-acetate pH 7.4

ii) 1 mM $MnCl_2$

iii) 10X native dye

iv) Agarose

v) 40 mM Tris acetate, pH 7.4

b) Miscellaneous: micropipettes, autoclaved micro tips, distilled water

c) Instruments: Weighing machine, microwave oven, agarose gel electrophoresis set up- tray, cassette, combs, lid, and power pack

For visualisation of gel: Gel documentation system with CCD camera

Method

a) The reaction mixture was prepared as follows and left to incubate for 60 min at room temperature-

S. No.	Components	Volume (µl)
1.	$MnCl_2$ (10 mM)	2 µl
2.	Tris Acetate (20 mM)	2 µl
3.	DNA	1 µl
4.	Protein (1 µM)	0-3 µl
5.	H_20	Rest of the volume
	Total	20 µl

b) 2 µl of 10X native dye was further added and the complexes resolved using 2% agarose gel electrophoresis for 5 hours at 50 volts in 40 mM tris acetate buffer, pH 7.4.

CHAPTER 4

RESULTS AND DISCUSSIONS

4.1 Primer design and amplification of DR_1143 gene

Study of any unknown gene function needs its cloning and expression of protein then one is able to study the function. We amplified the DR1143 gene using polymerase chain reaction (PCR). The forward and reverse primers for the PCR amplification were designed using gene DNA sequence of DR1143 gene. The primers were designed such that they both have the same melting temperature (55±5℃) and one unique restriction site was introduced in each primer for its cloning in the expression vector **(Figure 10)**. The PCR was performed using a high-fidelity DNA polymerase to avoid any sequence errors. The DR1143 gene was amplified from the *D. radiodurans* genome. The amplified product was resolved on 1% agarose gel. The appearance of a single expected size band on the gel confirms amplification of the gene. As shown in **Figure 11,** we got a single band of 1kb size that confirmed the amplification of DR1143 ORF. To remove the impurities and unutilized primers and nucleotides which interfere in the next experimental step the amplified product was purified as per protocol given in Material and Methods section **3.4.3.**

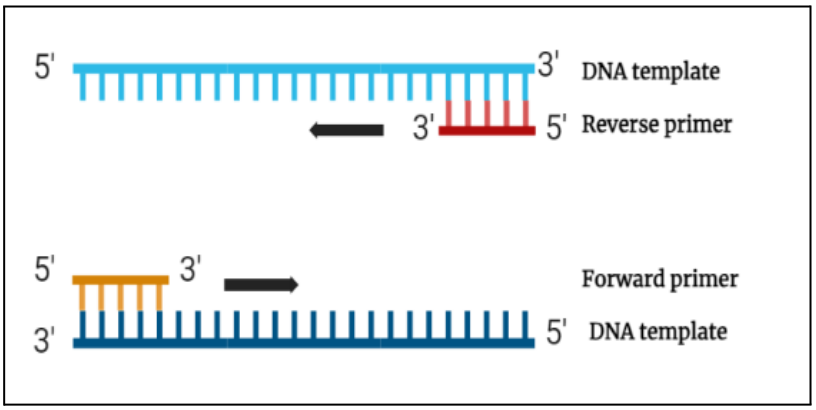


Figure 10. Primer design: The schematic representation shows the gene and forward and reverse primer binding location on the template DNA sequence

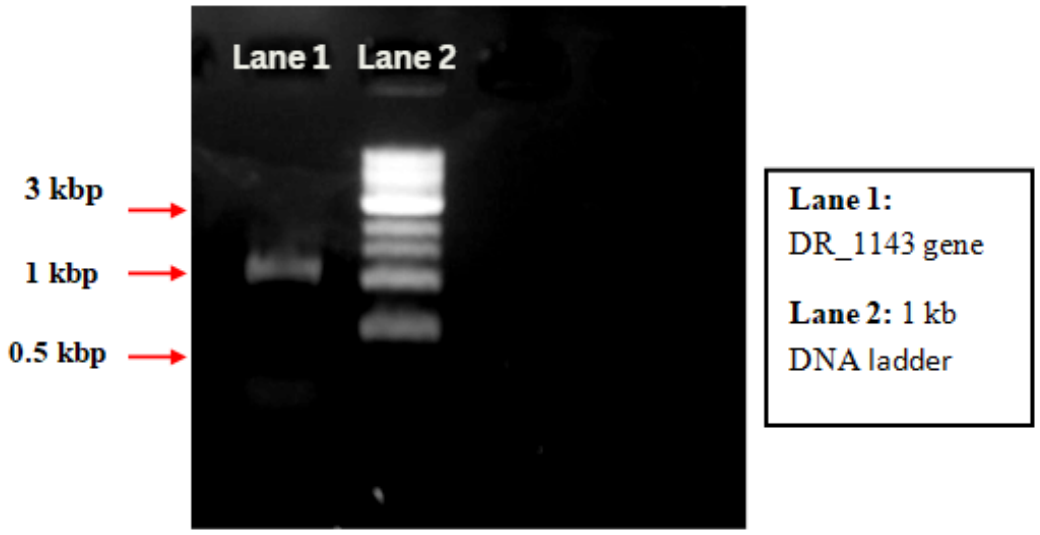


Figure 11. PCR amplification of DR_1143 gene: DR_1143 gene was PCR amplified from the genome of *Deinococcus radiodurans* R1 using a specific primer set and resolved on 1% agarose gel. Expected band size of ~1 kb confirms the amplification.

4.2 Cloning of DR_1143 gene in pTWIN1 plasmid

To clone the PCR amplified gene in a plasmid vector both the gene and plasmid need to be digested with the same restriction endonucleases. For cloning of PCR amplified DR_1143 in pTWIN1 expression vector both were digested with *EcoRI* and *BamHI* restriction endonucleases since both enzymes produce coherent DNA ends, it helps in directional cloning. The digestion was confirmed by resolving the products on the 1% agarose gel. The appearance of the linear DNA of plasmid with expected size and single 1kb band of DR_1143 confirms the restriction digestion **(Figure 12)**. Double digestion of the pTWIN1 plasmid with EcoRI and BamHI yielded two bands of sizes corresponding to ~6 kb and ~1 kb band, the lower band corresponding to previously cloned protein tag which gets digested out from plasmid. The DNA bands from the gel were extracted using the gel purification method as described in the Material and Methods section **3.5.1**. The gel-purified pTWIN1 plasmid and DR1143 gene were ligated using a DNA ligation kit consisting of T4 DNA ligase as described in the Material and Methods section **3.6**.

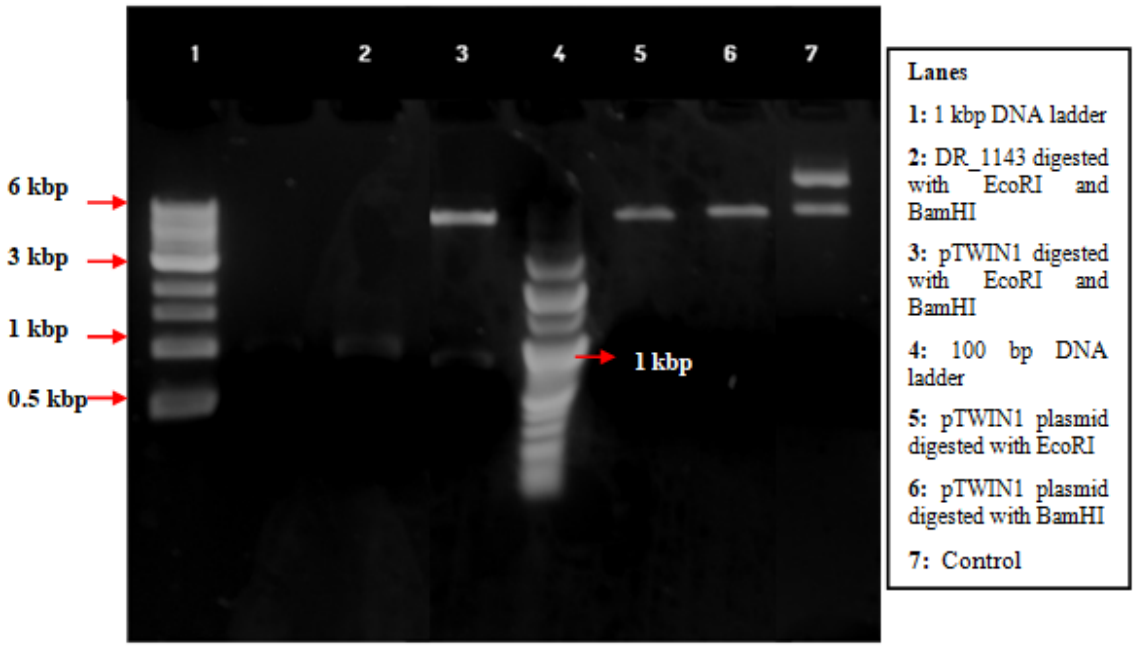


Figure 12. Restriction digestion of DR_1143 gene and pTWIN1 plasmid: Restriction digestion of PCR purified DR_1143 and pTWIN1 plasmid with EcoRI and BamHI enzymes. The digestion was confirmed by resolving the digested products on the agarose gel.

The ligation of DR1143 gene in pTWIN1 yields the pTWIN1-DR_1143 genetically modified recombinant vector **(Figure 13)** was then used for further transformation experiments. The schematic vector map shows the exact position of DR1143 in the plasmid and various restriction sites and antibiotic markers.

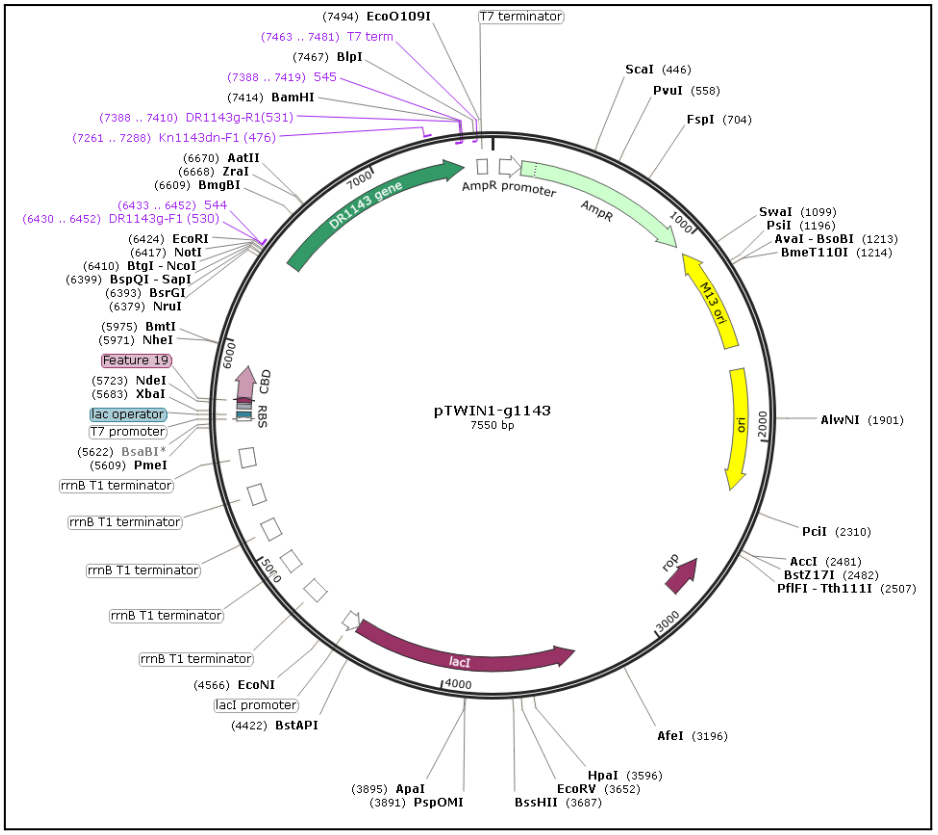


Figure 13: Schematic representation of pTWIN1-DR_1143 genetically modified vector

4.3 Transformation of pTWIN1- DR_1143 vector into *E. coli* DH5α cells

The DR1143 ligated recombinant vector pTWIN1- DR1143was transformed into competent *E. coli* DH5α cells. The positive clones were selected on LB agar plates containing carbenicillin antibiotic (100µg/ml) as selection pressure. After 15-16 hours of incubation, about 10 randomly picked-up colonies were patched on fresh LB agar plates containing the same amount of antibiotic and grown overnight. These colonies were used for colony PCR to confirm cloning. The plate was stored at 4°C for further use. The cloning was confirmed by colony PCR, restriction digestion, and finally by Sanger Sequencing.

4.3.1. Confirmation of cloning by colony PCR

The patched colonies were screened for positive clones. For this purpose, a colony PCR was performed for 10 randomly selected well-grown colonies. To determine if the

construct contains the DNA fragment of correct molecular size and orientation, the colony PCR was performed using a vector-specific forward primer and insert-specific reverse primer (DR_1143-R). The amplification product was resolved by performing agarose gel electrophoresis as shown in **figure 14**. Out of all the randomly selected colonies, 4th, 5th, and 7th colonies showed a single and expected-sized bands of ~1 kb. The result indicated that the DR_1143 gene has been successfully cloned in the pTWIN1 plasmid. These positively transformed colonies were selected for further experiments. These colonies were grown in LB broth and plasmid was isolated.

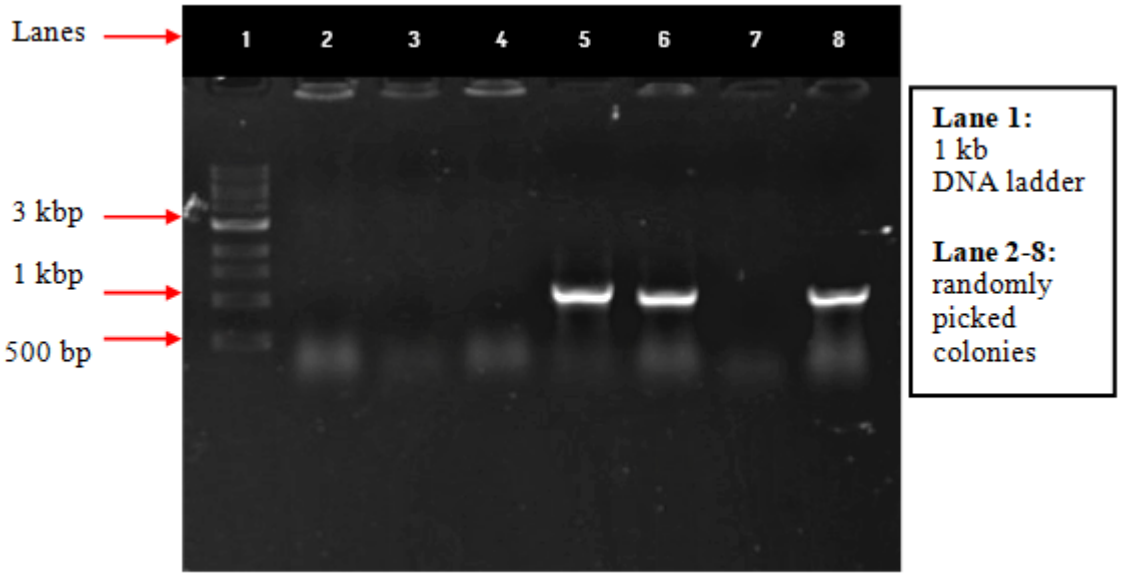


Figure 14: Cloning confirmation by Colony PCR: Colony PCR of transformed *E.coli* DH5α clones using pTWIN1 Forward and DR_1143 reverse primer set and resolved on 1% agarose gel. Expected bands of ~1 kb in 4th, 5th and 6th colonies showed the successful cloning of DR_1143 gene in the pTWIN1 plasmid.

4.3.2 Confirmation of cloning by Restriction digestion

The second confirmation of positive clones was performed by restriction digestion of recombinant plasmid to release the "insert out" using the same restriction enzymes used for cloning i.e. *EcoRI* and *BamHI*. Upon agarose gel electrophoresis, the colonies showed

bands of ~6 kb and ~1 kb sizes which correspond to pTWIN1 and DR_1143 respectively as shown in **figure 15**. The plasmid controls were also set up to check if the restriction enzymes were working properly. The controls showed 2-3 bands indicating the supercoiled, relaxed, and linear plasmid conformations. The results suggested that the restriction enzymes were working and the "insert release" of the correct size confirmed that the selected bacterial colonies bearing the DR1143 gene.

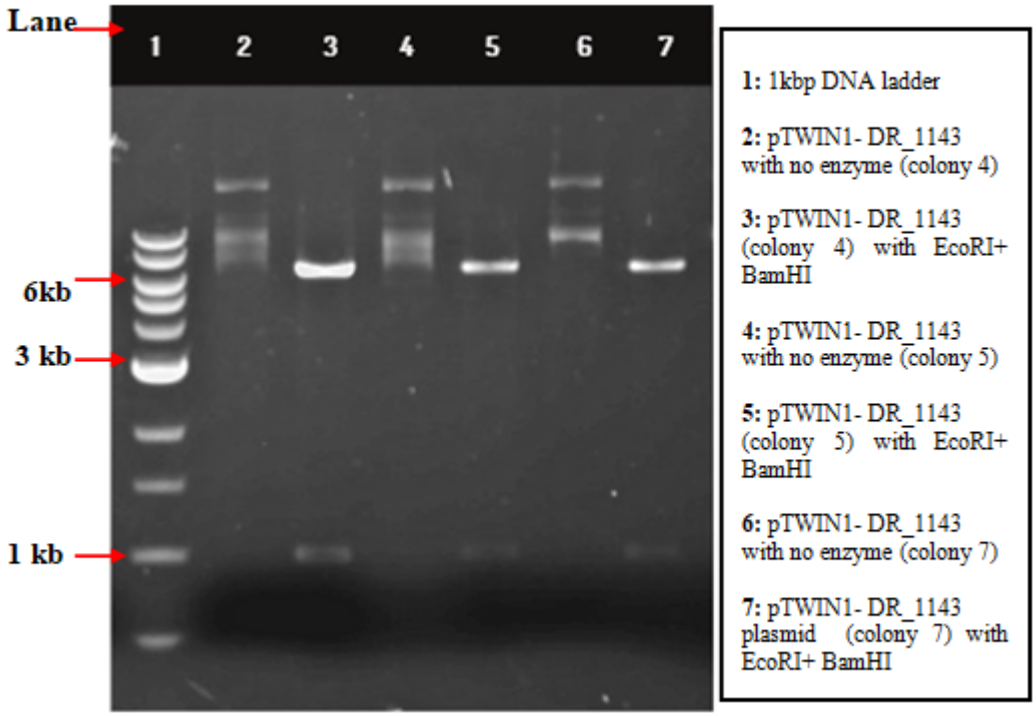


Figure 15: Cloning confirmation by Restriction digestion: Confirmation of DR_1143 cloned in pTWIN1 by restriction digestion with EcoRI and BamHI for insert release and resolved on 1% agarose gel.

4.3.3 Confirmation of cloning by Sanger sequencing

Although colony PCR and restriction digestion suggested the cloning and construction of the pTWIN1-DR1143 recombinant vector, these techniques cannot detect the point mutations if there are any in the DNA sequence. Sequencing verifies the insert's DNA sequence and orientation and enables examination of the junctions between the insert and plasmid DNA. Therefore, to confirm the correctness of the DNA sequence of the cloned

DNA, we performed Sanger DNA Sequencing (outsourced). The results showed that there are no errors in the DNA sequence, thus confirming the successful construction of the pTWIN1-DR1143 plasmid.

4.4 In-silico analysis:

There are many online tools available for predicting the structure and function of uncharacterized proteins, eg. BLAST which does similarity search with database **(figure 16)**, phyre[2] which searches for structural similarity. The results suggest that it shows more structural similarity with DNA binding proteins. Some of the bioinformatics results are represented below.

4.4.1 BLASTp results

Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
hypothetical protein [Deinococcus hopiensis]	Deinococcus hopiensis	494	494	99%	4e-172	80.31%	325	WP_084049739.1
hypothetical protein [Deinococcus betulae]	Deinococcus betulae	477	477	96%	8e-166	78.55%	321	WP_224571700.1
hypothetical protein [Deinococcus arboris]	Deinococcus arboris	477	477	96%	2e-165	78.23%	321	WP_198170408.1
hypothetical protein [Deinococcus arcticus]	Deinococcus arcticus	458	458	96%	3e-158	78.23%	321	WP_199188295.1
hypothetical protein [Deinococcus aquaedulcis]	Deinococcus aquaedulcis	452	452	96%	1e-155	78.23%	321	WP_221088447.1
TPA: hypothetical protein [Cyanobacteria bacterium UBA11049]	Cyanobacteria bacterium UBA11049	245	245	99%	4e-75	42.28%	329	HCF28315.1
hypothetical protein [Nostocales]	Nostocales	240	240	96%	3e-73	39.05%	327	WP_190676285.1
hypothetical protein [Nostocaceae]	Nostocaceae	240	240	96%	4e-73	39.05%	327	WP_190591398.1
TPA: hypothetical protein [Cyanobacteria bacterium UBA11162]	Cyanobacteria bacterium UBA11162	240	240	98%	5e-73	39.63%	335	HBL10544.1
hypothetical protein NIES4101_87910 [Calothrix sp. NIES-4101]	Calothrix sp. NIES-4101	237	237	97%	3e-72	39.38%	326	BAZ42821.1
TPA: hypothetical protein [Cyanobacteria bacterium UBA12227]	Cyanobacteria bacterium UBA12227	237	237	98%	8e-72	39.32%	335	HAG85640.1
hypothetical protein [Nostocaceae]	Nostocaceae	235	235	97%	2e-71	40.00%	327	WP_011320094.1
hypothetical protein [Cyanomargarita calcarea GSE-NOS-MK-12-04C]	Cyanomargarita calcarea GSE-NOS-MK-12-04C	232	232	97%	3e-70	38.24%	326	MBW4667712.1
hypothetical protein [Coleofasciculus sp. S288]	Coleofasciculus sp. S288	232	232	98%	4e-70	40.19%	333	MCA1992383.1
hypothetical protein A6S26_12560 [Nostoc sp. ATCC 43529]	Nostoc sp. ATCC 43529	231	231	98%	9e-70	39.25%	327	RCJ28351.1
hypothetical protein [Nostoc]	Nostoc	231	231	98%	1e-69	39.25%	327	WP_190953890.1
hypothetical protein [Symplocastrum sp. BBK-W-15]	Symplocastrum sp. BBK-W-15	231	231	99%	1e-69	37.04%	328	WP_254012343.1

Figure 16 . BLASTp results showing protein sequences similar to that of protein encoded by DR_1143 gene

Organisms with maximum query coverage and percent identity higher than 35% are shown in **figure 17.**

Organism	Query cover	% Identity
Deinococcus hopiensis	99%	80.31%
Cyanobacteria bacterium UB11049	99%	42.285%
Coleofasciculus sp. S288	98%	40.19%
Nostoc sp. TCC 43529	98%	39.25%
Symplocastrum sp. BBK-W-15	99%	37.04%

Figure 17: Organisms with maximum query coverage and percent identity higher than 35%

4.4.2 Predicted tertiary structure of protein encoded by DR_1143 gene

The 3-D structure of DR_1143 protein, as predicted by Phyre[2] is shown in **figure 18 .** The query protein is of 317 AA residues. The confidence and coverage of structure is 4.3% and 97% respectively.

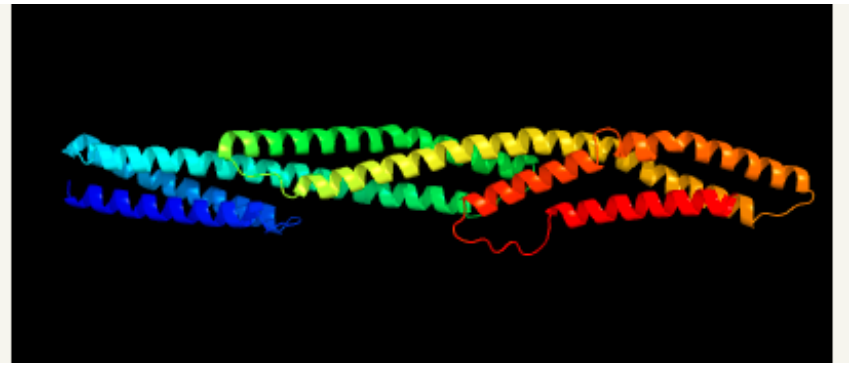


Figure 18: A coil like protein tertiary structure of protein encoded by DR_1143 gene, predicted by phyre[2] server

4.5 Transformation of the pTWIN1-DR_1143 recombinant vector into BL21-Codon Plus-RIL cells and overexpression of DR_1143 protein

The confirmed plasmids were further transformed into *E. coli* overexpression strain BL21- Codon Plus - RIL cells for over-expression of DR_1143 protein using an IPTG inducer. BL21-CodonPlus cells can express several recombinant genes at high levels that

are not possible in standard BL21 strains due to the extra tRNAs that are available. IPTG, also known as isopropyl β-D-1-thiogalactopyranoside, is a chemical analog of allolactose that induces gene expression by removing a repressor from the lac operon. The transformed cells along with negative control (pTWIN1 plasmid) were grown till the OD_{600} reaches 0.5 (cells are at the log phase of the growth curve). After IPTG addition (0.5 mM), the culture was incubated overnight at 20° C. Induction at 20° C instead of 37° C reduces growth rate, as a result, the proteins are expressed slowly and they get proper time for folding. This helps to prevent the formation of inclusion bodies.

The whole cell protein was extracted by lysing the cells by sonication. To prevent protein degradation, all the steps were performed on ice. The whole cell protein was resolved on 12% SDS-PAGE (**figure 19**). The appearance of an intense band corresponding to 35 kDa in the test sample and no band in the control confirms the DR_1143 protein over-expression. Maximum protein appeared in the clarified cell extract indicating the protein is present in a soluble form.

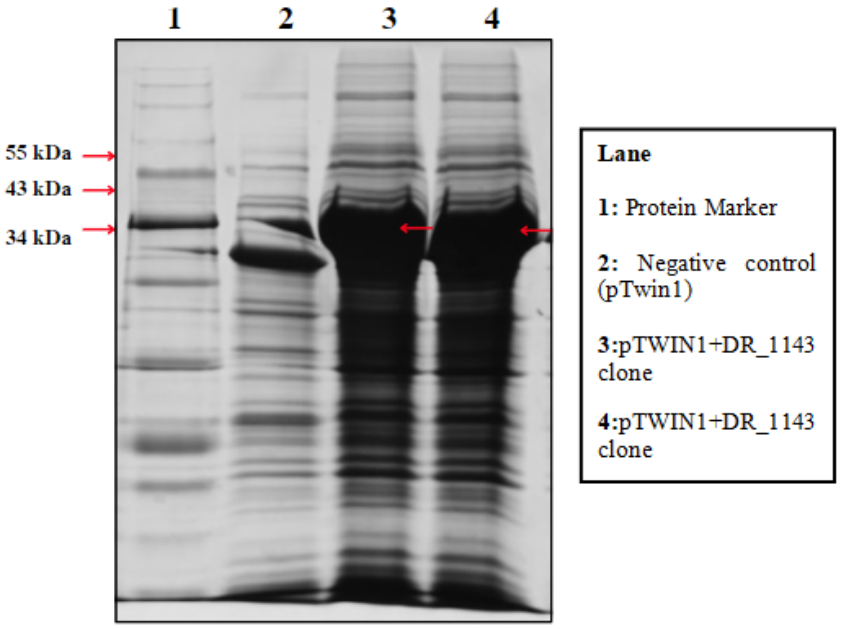


Figure 19. Overexpression of DR_1143 protein: DR_1143 protein overexpression was induced by IPTG. The protein was extracted after lysing the cells by sonication. The arrows in lanes 3 and 4 (←) show the induced protein bands on the gel corresponding to ~35 kDa confirming the protein overexpression.

4.6 DR_1143 protein purification

4.6.1 Affinity Chromatography

The DR_1143 protein purification was carried out by Affinity chromatography using Chitin resin. The IMPACT-TWIN system is a protein purification technique that uses the inducible self-cleavage activity of protein splicing components (referred to as inteins) to separate the target protein from the affinity tag. A target protein's (DR_1143) N-terminus can be fused with the *Ssp*DnaB (intein 1) self-cleavable intein-tag to enable protein purification with a pH adjustment without the need for thiol reagents. The Chitin Binding domain present in the intein tag has a high affinity for chitin beads leading to protein purification.

The clarified cell extract (supernatant) was passed through the chitin column after equilibration of the column with column buffer (20 mM Tris-HCl, pH 8.5, 0.5 M NaCl) to maintain a constant pH in the column. The column was washed twice with the same buffer to remove all the unbound proteins. Loading and washing at high NaCl concentrations reduces the non-specific protein binding. For the elution of the target protein, 20 mM sodium phosphate buffer, pH 6.0, 0.5 M NaCl was added to the column. Analysis of the clarified cell extract, flow-through, wash 1, wash 2, matrix, and two eluents fractions through 12% SDS- PAGE indicated the appearance of intense protein bands corresponding to 35 kDa in the crude, matrix, and eluent fraction while no bands were observed in the washes as shown in **figure 20** .

.

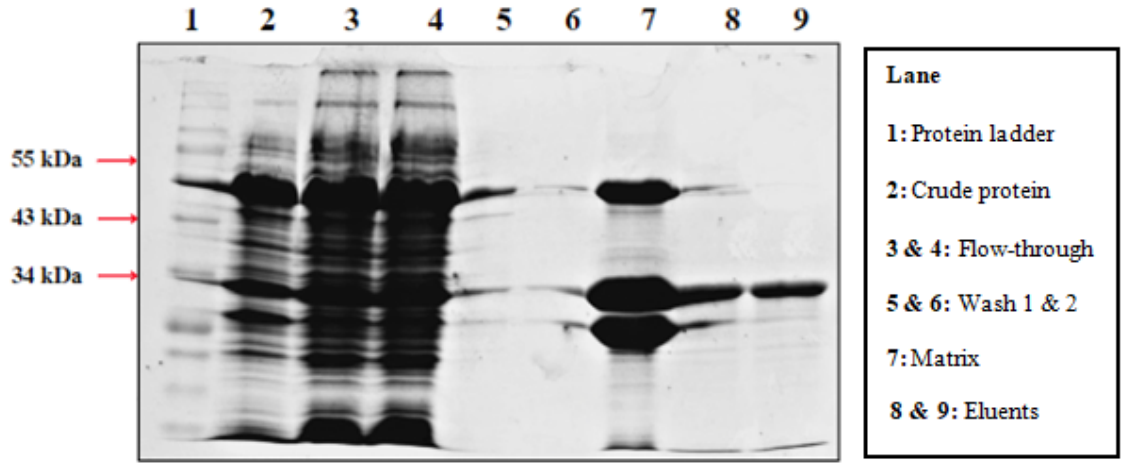
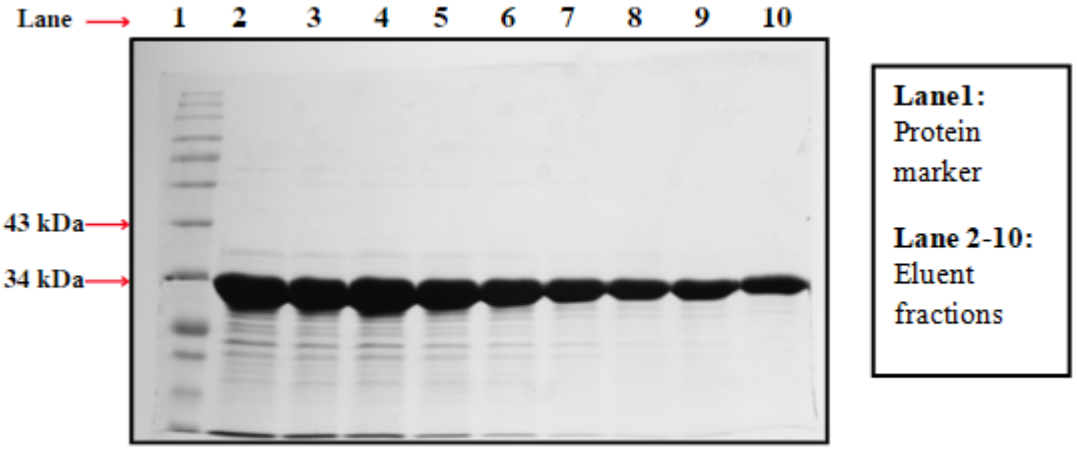


Figure 20: Purification of DR_1143 protein by affinity chromatography: The DR_1143 protein was purified by affinity chromatography using chitin resin. Intense protein bands corresponding to 35 kDa appeared in crude, matrix and eluents fraction, resolved by 12% SDS- PAGE.

After saturation of the column with the cleavage buffer (change of pH paper strip colour to yellow), the column was incubated overnight at 4° C. Analysis of the different fractions of the eluents by 12% SDS- PAGE (**figure 21**) indicated the appearance of protein bands corresponding to 35kDa in all the fractions. However, multiple small bands were also observed along with the expected-sized bands.



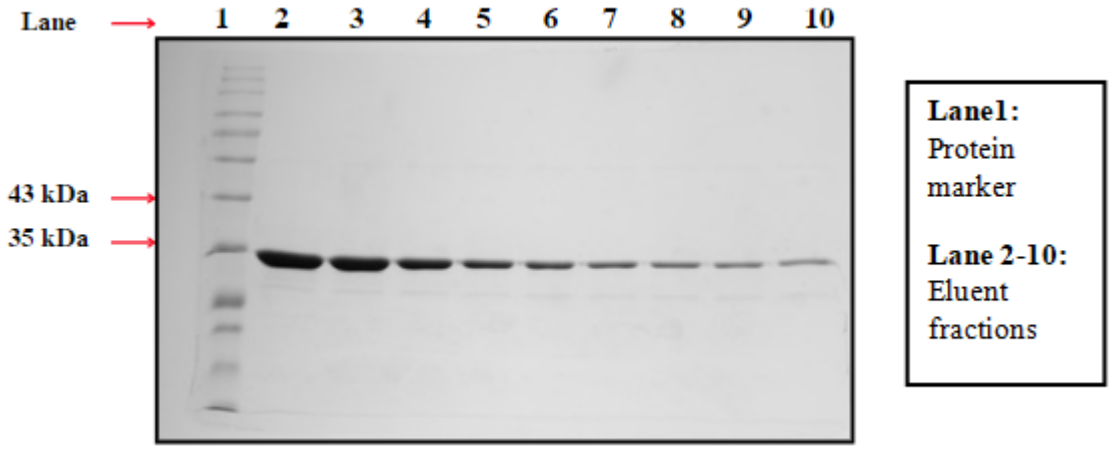


Figure 21. Elution of DR_1143 protein: The target protein DR_1143 was eluted after the addition of cleavage buffer (20 mM sodium phosphate buffer, pH 6.0, 0.5 M NaCl). Expected sized bands corresponding to 35 kDa appeared in all the 18 eluents fractions, resolved by 12% SDS- PAGE.

The NaCl concentration in the samples was reduced by dialysis using a 20 mM Tris- HCl, pH 8.0, and 25 mM NaCl buffer. By running the purified fractions via Q-sepharose anion exchange chromatography, any remaining trace amounts of contaminating proteins were eliminated.

4.6.2 Q-Sepharose Anion Exchange Chromatography

Q- Sepharose Fast Flow is an ion exchange chromatography resin with the quaternary amine functional group [$-CH_2-N+(CH_3)_3$] functioning as a strong anion exchanger. At pH above their pI, protein would carry a negative charge and will bind to the resin. A NaCl gradient from 25 mM to 500 mM was used to elute the column. With the increasing salt concentration, negatively charged chloride (Cl^-) ions would bind to the resin, and the proteins were eluted.

Analysis of the various fractions of input, flow-through, wash, and eluents by 12% SDS-PAGE revealed the appearance of intense protein bands corresponding to 35 kDa in the input and 9-16 fractions of the eluents (**figure 22**). These protein fractions were pooled together, dialyzed overnight using storage buffer (20mM tris pH 8.3, 500 mM NaCl, 1 mM EDTA, 1 mM β-Me, 50% glycerol) and stored at -70° C until further use.

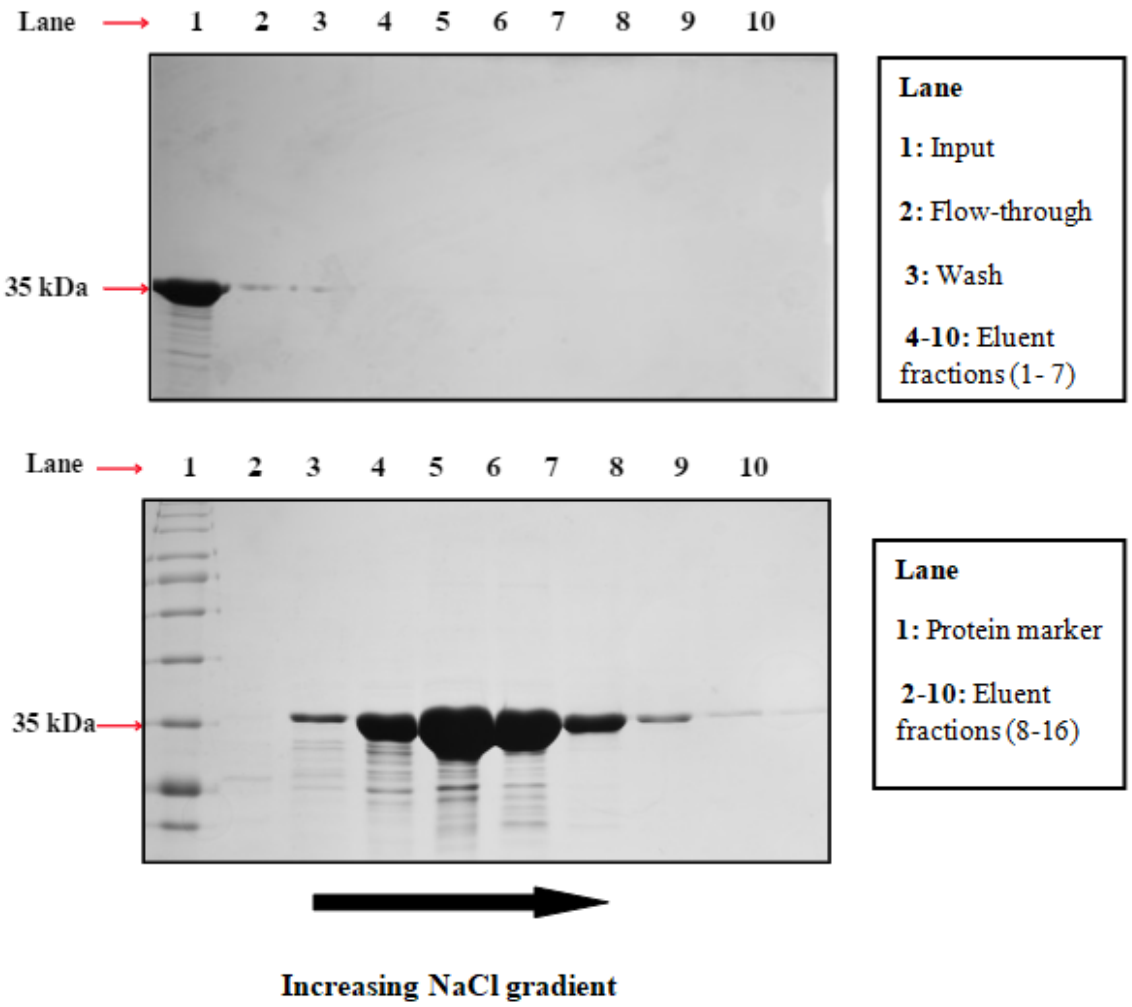


Figure 22. DR_1143 protein purification by Q-Sepharose anion exchange chromatography: Q- Sepharose Fast Flow bears a quaternary amine functional group which functions as a strong anion exchanger. A NaCl gradient from 25 mM to 500 mM was used for protein elution. Analysis of the input, flow-through, wash, and eluents fractions by 12% SDS-PAGE revealed intense protein bands corresponding to 35 kDa in the fractions eluted later (9-16 fractions).

4.7 Protein estimation by Folin's Lowry Method

The Folin's Lowry method was used to estimate the concentrations of the various purified protein fractions using BSA (1 mg/ml) as standard solution. The absorbance was measured at 760 nm. The addition of deoxycholate and trichloroacetic acid further helps in protein precipitation. From the standard graph, the concentrations of the protein

fractions F1, F2, F3, F4, F5 were found out to be 3.561, 4.63, 3.715, 2.54 and 3.01 µg/µl respectively **(Figure 23)**.

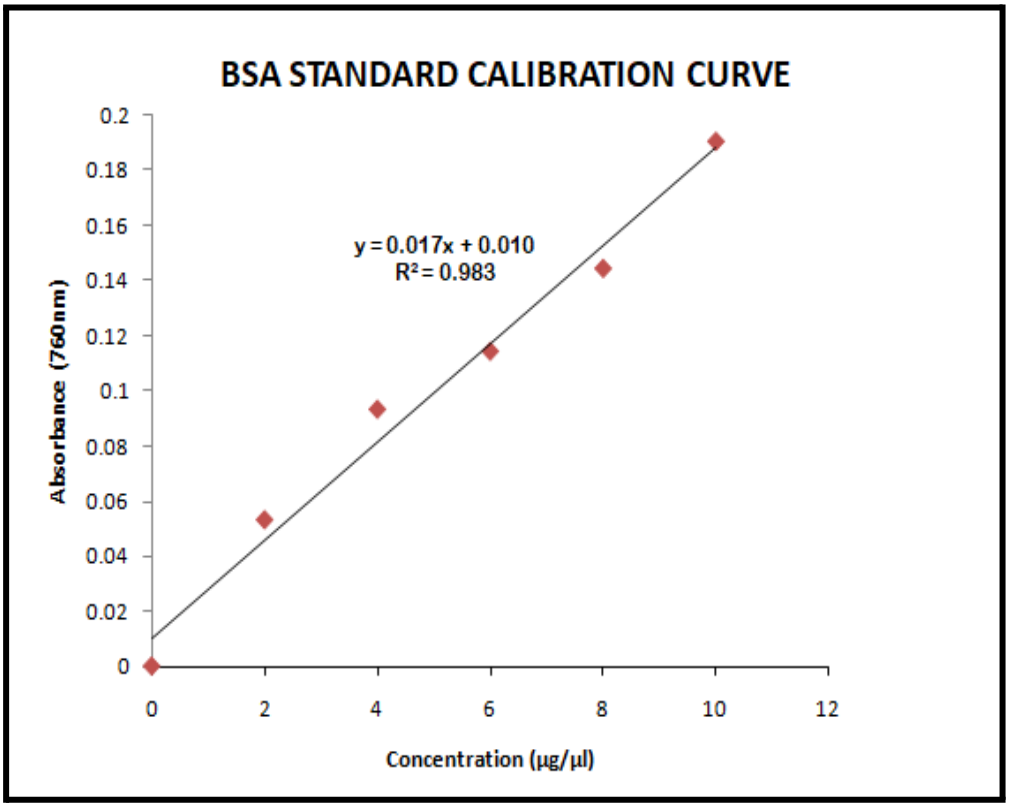


Figure 23. BSA standard calibration curve: The concentrations of various protein fractions were estimated by the Folin -Lowry method and the absorbance measured at 760 nm.

4.8 DR_1143 protein oligomeric status determination by glutaraldehyde cross-linking

The oligomeric status of DR_1143 protein was determined by glutaraldehyde cross linking method. When distance between 2 protein subunits is ≤ 5 Å, glutaraldehyde cross links between them, as a result, the molecular weight increases leading to bands of different sizes in the control and test samples. As shown in **figure 24**, upon cross-linking with glutaraldehyde, the DR_1143 protein is forming a multimer. On dividing the

molecular weight of the polymer i.e. ~250 kDa with the control (~35 kDa), it can be inferred that the aggregate is a polymer consisting of ~7 monomeric units.

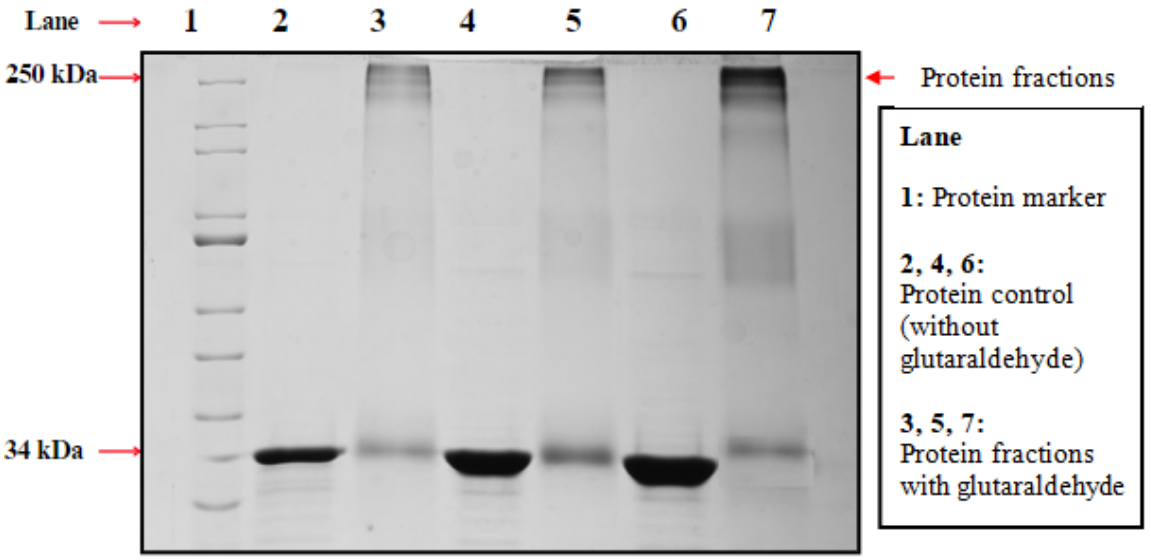


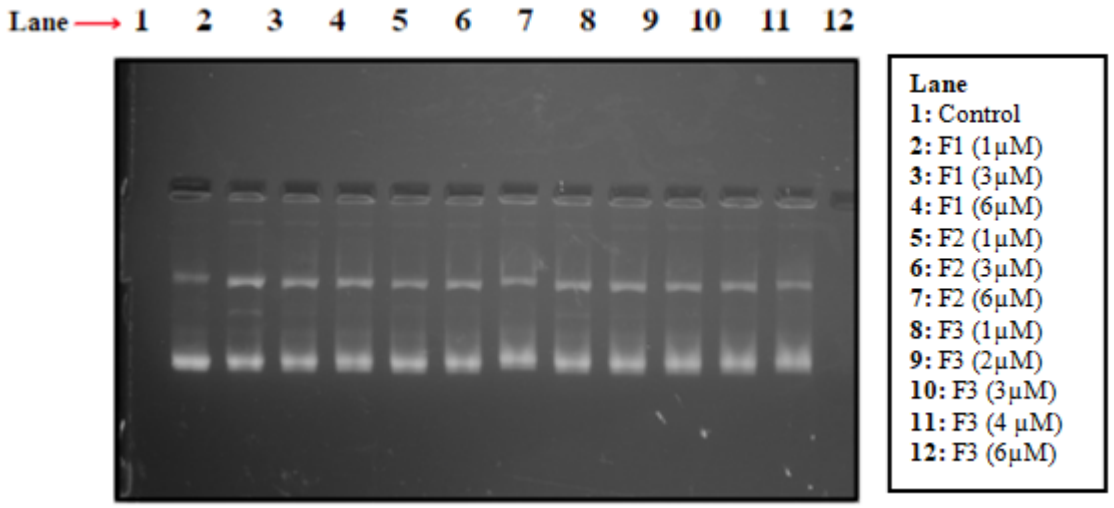
Figure 24. Oligomeric status of DR_1143 protein: The oligomeric status of DR_1143 protein was determined by glutaraldehyde cross linking method. Upon cross-linking with glutaraldehyde the protein formed a multimer of ~250 kDa .

4.9 Biochemical characterization of DR_1143 protein

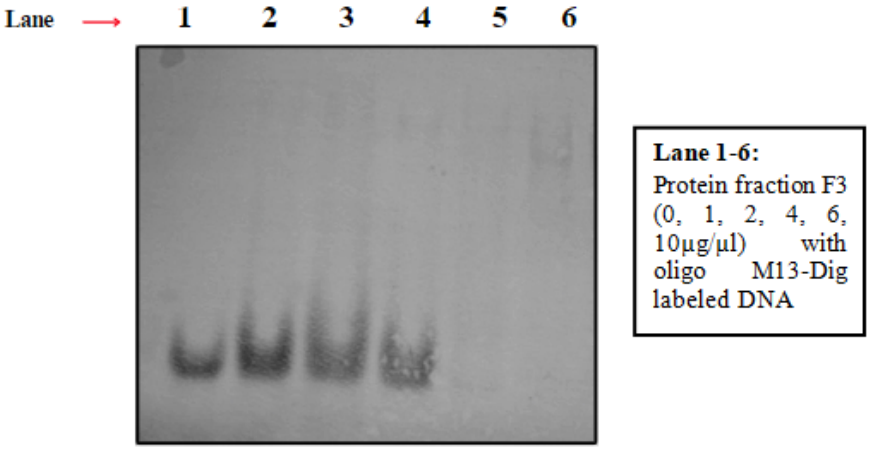
Electrophoretic Mobility Shift Assay:

The DNA binding activity of DR_1143 protein was evaluated by Electrophoretic Mobility Shift Assays (EMSA) using oligo DT50-DIG, oligo-M13-DIG, single and double stranded DNA of ΦX174 virion at a range of protein concentrations.. No significant binding of DR_1143 protein was observed with double stranded DNA of ΦX174 virion, oligo M13-Dig and DT50-DIG labelled DNA **(figure 25)** .

(A)


Lane → 1 2 3 4 5 6 7 8 9 10 11 12
Lane
1: Control
2: F1 (1µM)
3: F1 (3µM)
4: F1 (6µM)
5: F2 (1µM)
6: F2 (3µM)
7: F2 (6µM)
8: F3 (1µM)
9: F3 (2µM)
10: F3 (3µM)
11: F3 (4 µM)
12: F3 (6µM)

(B)


Lane → 1 2 3 4 5 6
Lane 1-6:
Protein fraction F3 (0, 1, 2, 4, 6, 10µg/µl) with oligo M13-Dig labeled DNA

(C)

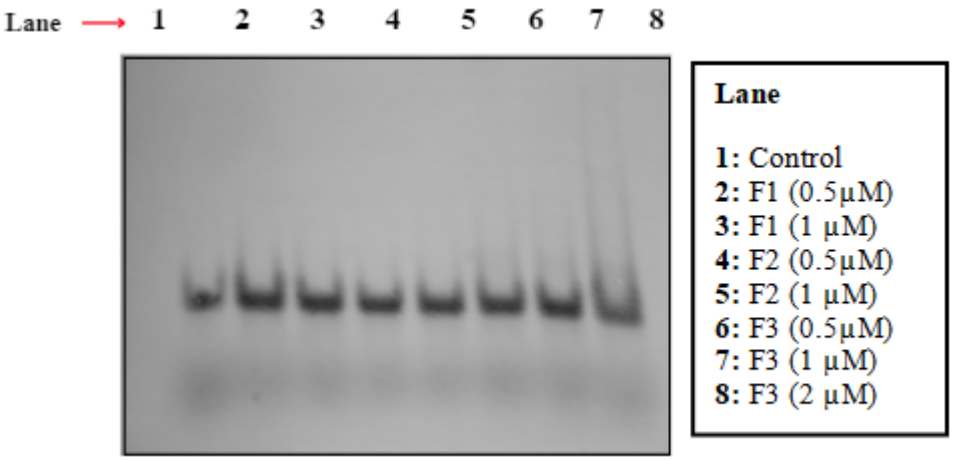


Figure 25: DNA-DR_1143 Protein binding studies with (A) Double stranded DNA of ΦX174 virion (5386 bp), (B) oligo M13-Dig labelled DNA (67 mer), and (C) oligo DT-50 Dig labelled DNA: EMSA studies showed no significant DNA- protein binding with all the 3 DNA substrates.

Among the various DNA substrates used, DNA-Protein binding was observed only with single stranded DNA of ΦX174 virion **(figure 26).** The binding increased with increasing protein concentrations while no DNA binding was observed with BSA (control).

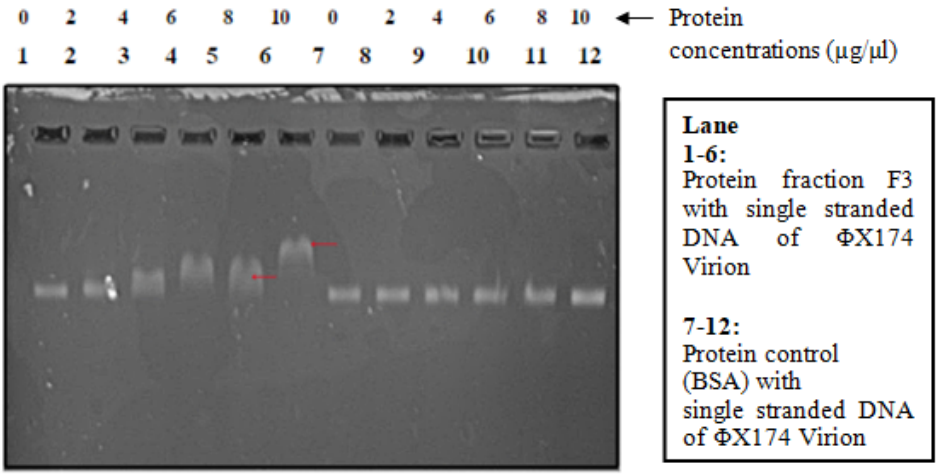


Figure 26: DR_1143 protein binding with single stranded DNA of ΦX174 virion: EMSA studies showed DNA-Protein binding with single stranded DNA of ΦX174 virion

(5386 nucleotides). The (←) arrows indicate DNA: protein binding complexes and resolved by 2% agarose gel electrophoresis.

From EMSA studies, it can be inferred that the DR_1143 protein binds to longer single stranded DNA and not smaller molecules. Literature analysis revealed that the DR_1143 protein bears similarity to a group of proteins known as SSBs. SSBs bind to single stranded DNA molecules formed during DNA unwinding and play an important role in various biological processes. ssDNA is more vulnerable to chemical and nucleolytic assaults that might harm the genome and is less thermodynamically stable than dsDNA. SSBs recruit several DNA metabolic enzymes to the ssDNA, bind to it in a sequence-independent manner to protect it from nucleolytic destruction, and induce the DNA damage cell cycle checkpoint response in eukaryotes. They contribute to the formation of the main nucleoprotein complex substrate during DNA replication, recombination, and repair processes. Some examples of SSBs include RecA, RPA and priB.

CHAPTER 5

SUMMARY AND FUTURE PROSPECTS

Deinococcus radiodurans is an extremely radioresistant bacterium, characterized by an exceptional ability to withstand the lethal effects of DNA damaging agents including ionizing radiation, UV radiation, desiccation and many chemical mutagens. The resistance of the organism is derived from its error free DNA repair mechanism. It can repair thousands of SSBs and hundreds of DSBs generated by high doses of radiation. This unique feature has made *D. radiodurans* a model for studying the different genes and their corresponding proteins involved in maintaining DNA integrity and stability. DR_1143 is one of the uncharacterized genes induced upon radiation and several genotoxic stresses. We hypothesise that DR_1143 may be involved in the radiation resistance mechanism of *D. radiodurans.* Therefore, this gene was studied in detail.

In the present study, the DR_1143 gene was successfully amplified from the genome using a gene specific primer and cloned in pTWIN1 (expression vector) by T4 DNA Ligase. The recombinant vector pTWIN1-DR_1143 was transformed into *E. coli* DH5α cells. After confirmation of cloning by colony PCR, restriction digestion, and Sanger sequencing, the confirmed plasmids were transformed into BL21-Codon Plus-RIL cells (*E.coli* overexpression strain). The protein over expression was induced by using IPTG. The whole cell protein was extracted by lysing the cells by sonication. We got the protein in soluble form which is important for its purification. The protein purification was carried out by affinity chromatography using chitin beads and Q-Sepharose anion exchange chromatography. After two step purification the protein was purified near to homogeneity. The yield of the protein was very high, 5mg/ml. It was observed that this protein is forming a multimer as it was seen in the higher size band in native gel and even with the glutaraldehyde crosslinking method. Biochemical characterization of the protein was carried out by EMSA studies which showed protein binding with single stranded DNA of ΦX174 virion revealing its similarity to single stranded DNA Binding proteins (SSB) which play an important role during DNA replication and repair processes. Further studies needed to fully understand this protein functions like its interactions with other proteins.

CHAPTER 6

BIBLIOGRAPHY

1. Albuquerque L, Simões C, Nobre MF, Pino NM, Battista JR, Silva MT, Rainey FA, de Costa MS (2005). Truepera radiovictrix gen. nov., sp. nov., a new radiation resistant species and the proposal of Trueperaceae fam. nov. *FEMS micro. Let.* 247: 161-169.
2. Anderson R and Hansen K (1985). Structure of a novel phosphoglycolipid from Deinococcus radiodurans. *Jour. of Bio. Chem.* 260: 12219-12223.
3. Anderson AW (1956). Studies on a radio-resistant micrococcus. I. Isolation, morphology, cultural characteristics, and resistance to gamma radiation. *Food Tech*. 10: 575-577
4. Awile O, Krisko A, Sbalzarini IF and Zagrovic B (2010). Intrinsically disordered regions may lower the hydration free energy in proteins: a case study of nudix hydrolase in the bacterium Deinococcus radiodurans. *PLoS Comput. Bio.* 6: e1000854.
5. Basu B and Apte SK (2012). Gamma radiation-induced proteome of Deinococcus radiodurans primarily targets DNA repair and oxidative stress alleviation. *Mol. Cell Proteo*. 11: 1.
6. Bauermeister AE, Bentchikou R, Moeller and Rettberg P (2009). Roles of PprA, IrrE, and RecA in the resistance of Deinococcus radiodurans to germicidal and environmentally relevant UV radiation. *Arch. Microbiol.* 191: 913–91
7. Bonacossa AC, Coste G, Sommer S and Bailone A (2002). Quantification of RecA protein in Deinococcus radiodurans reveals involvement of RecA, but not LexA, in its regulation. *Mol. Gen. Genom*. 268: 28–41.
8. Brim H, McFarlan SC, Fredrickson JK, Minton KW, Zhai M, Wackett LP and Daly MJ (2000). Engineering Deinococcus radiodurans for metal remediation in radioactive mixed waste environments. *Nat. Biotechnol.* 18:85–90.
9. Cox MM and Battista JR (2005). Deinococcus radiodurans—the consummate survivor. *Nat. Rev. Microbiol.* 3:882–892.
10. Daly MJ, Gaidamakova EK, Matrosova VY, Vasilenko A, Zhai M, Leapman RD, Lai B, Ravel B, Li SMW, Kemner KM and Fredrickson JK (2007). Protein oxidation implicated as the primary determinant of bacterial radioresistance. *PLoS bio.*, *5*(4): e92.

11. Daly MJ, Gaidamakova EK, Matrosova VY, Kiang JG, Fukumoto R, Lee DY, Wehr NB, Viteri GA, Berlett BS, Levine RL (2010). Small-molecule antioxidant proteome-shields in Deinococcus radiodurans. *PloS one.* 5(9): e12570.
12. Dartnell LR, Hunter SJ, Lovell KV, Coates AJ, Ward JM (2010). Low-temperature ionizing radiation resistance of Deinococcus radiodurans and Antarctic Dry Valley bacteria. *Astrobio*. 10(7): 717-32.
13. De Groot A, Dulermo R, Ortet P, Blanchard L, Guérin P, Fernandez B, Vacherie B, Dossat C, Jolivet E, Siguier P, Chandler M (2009). Alliance of proteomics and genomics to unravel the specificities of Sahara bacterium Deinococcus deserti. *PLoS Gen.* 5(3): e1000434.
14. Driedger AA (1970). The ordered growth pattern of microcolonies of Micrococcus radiodurans: first generation sectoring of induced lethal mutations. *Can. J. Microbiol.* 16: 1133–1135.
15. Duggan DE, Anderson AW and Elliker PR (1963). Inactivation of the radiation-resistant spoilage bacterium Micrococcus radiodurans. II. Radiation inactivation rates as influenced by menstruum temperature, preirradiation heat treatment, and certain reducing agents. *Appl. Microbiol.* 11: 413–417.
16. Ferreira AC, Nobre MF, Rainey FA, Silva MT, Wait R, Burghardt J, Chung AP, Da Costa MS. Deinococcus geothermalis sp. nov. and Deinococcus murrayi sp. nov., two extremely radiation-resistant and slightly thermophilic species from hot springs (1947). *Inter. Jour. of System. & Evol. Microbio.* 47(4): 939-947.
17. Fernández Zenoff V, Siñeriz F, Farias ME (2006). Diverse responses to UV-B radiation and repair mechanisms of bacteria isolated from high-altitude aquatic environments. *App. & environ. microbio*. 72(12): 7857-7863.
18. Franca MB, Panek AD and Eleutherio EC (2007). Oxidative stress and its effects during dehydration. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 146: 621–631.
19. Fredrickson JK, Li SM, Gaidamakova EK, Matrosova VY, Zhai M, Sulloway HM, Scholten JC, Brown MG, Balkwill DL, Daly MJ (2008). Protein oxidation:key to bacterial desiccation resistance?. *The ISME jour.* 2(4): 393-403.
20. Geisel N (2011). Constitutive versus responsive gene expression strategies for growth in changing environments. *PLoS One.* 6(11): e27033.

21. Gerard E, Jolivet E, Prieur D and Forterre P (2001). DNA protection mechanisms are not involved in the radioresistance of the hyperthermophilic archaea Pyrococcus abyssi and P. furiosus. *Mol. Genet. and genom.* 266: 72-78.
22. Girard AE (1971). A comparative study of the fatty acids of some micrococci. *Can. J. Microbiol.* 17: 1503–1508.
23. Gladyshev E and Meselson M (2008). Extreme resistance of bdelloid rotifers to ionizing radiation. *Proc. Natl. Acad. Sci. U. S. A.* 105: 5139–5144.
24. Gutman PD, Carroll JD, Masters CI and Minton KW (1994). Sequencing, targeted mutagenesis and expression of a recA gene required for the extreme radioresistance of Deinococcus radiodurans. *Gene.* 141: 31–37
25. Halliwell B and Gutteridge JM (2015). *Free radicals in biology and medicine*, 3rd ed. Oxford University Press, Oxford, United Kingdom.
26. Harsojo, Kitayama S and Matsuyama A (1981). Genome multiplicity and radiation resistance in Micrococcus radiodurans. *Jour. of biochem.* 90: 877-880.
27. Hawiger J and Jeljaszewicz J (1967). Antibiotic sensitivity of Micrococcus radiodurans. *Appl. Microbiol.* 15: 304–306.
28. He Y (2009). High cell density production of Deinococcus radiodurans under optimized conditions. *J. Ind. Microbiol. Biotechnol.* 36: 539–546.
29. Kitayama S (1982). Adaptive repair of cross-links in DNA of Micrococcus radiodurans. *Biochim. Biophys. Acta*. 697: 381– 384.
30. Kitayama S, Asaka S and Totsuka K (1983). DNA double-strand breakage and removal of cross-links in Deinococcus radiodurans. *J. Bacteriol.* 155: 1200–1207
31. Krisko A and Radman M (2010). Protein damage and death by radiation in Escherichia coli and Deinococcus radiodurans. *Proc. Natl. Acad. Sci. U. S. A.* 107: 14373–14377.
32. Lai WA, Kämpfer P, Arun AB, Shen FT, Huber B, Rekha PD, Young CC. Deinococcus ficus sp. nov., isolated from the rhizosphere of Ficus religiosa (2006). *Inter.l jour. of syst. and evolu. microbiolo.* 56(4): 787-791.
33. Makarova KS, Aravind L, Wolf YI, Tatusov RL, Minton KW, Koonin EV, Daly MJ (2001). Genome of the extremely radiation-resistant bacterium Deinococcus radiodurans viewed from the perspective of comparative genomics. Microbio. and Mole. Bio. Rev. 65(1): 44-79.

34. Makarova KS, Omelchenko MV, Gaidamakova EK, Matrosova VY, Vasilenko A, Zhai M, Lapidus A, Copeland A, Kim E, Land M, Mavromatis K (2007). Deinococcus geothermalis: the pool of extreme radiation resistance genes shrinks. *PLoS One.* 2(9): e955.
35. Martinez A, and Kolter R (1997). Protection of DNA during oxidative stress by the nonspecific DNA-binding protein Dps. *J. Bacteriol.* 179: 5188– 5194.
36. Mattimore V and Battista JR (1996). Radioresistance of Deinococcus radiodurans: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. *J. Bacteriol.* 178: 633–637.
37. Mennecier SGC, Servant P, Bailone A and Sommer S (2004). Mismatch repair ensures fidelity of replication and recombination in the radioresistant organism Deinococcus radiodurans. *Mol. Genet. Genom.* 272: 460–469.
38. Moseley BE and Setlow JK (1968). Transformation in Micrococcus radiodurans and the ultraviolet sensitivity of its transforming DNA. *Proc. Natl. Acad. Sci. U. S. A.* 61: 176–183.
39. Moseley BEB and Mattingly A (1971). Repair of irradiated transforming deoxyribonucleic acid in wild type and a radiation sensitive mutant of Micrococcus radiodurans. *J. Bacteriol.* 105: 976–983.
40. Murray RG, Hall M and Thompson BG (1983). Cell division in Deinococcus radiodurans and a method for displaying septa. *Can. J. Microbiol*. 29: 1412–1423.
41. Murray RG (1986). Family II. Deinococcaceae, pp1035–1043.
42. Murray, R.G., 1992. The family deinococcaceae. In: *The Prokaryotes: a handbook on the biology of bacteria: ecophysiology, isolation, identification, applications*, pp. 3732-3744.
43. Hess M (2003). Analysis of Deinococcus radiodurans mutants.
44. Narasimha A and Basu B (2021). New insights into the activation of radiation desiccation response regulon in Deinococcus radiodurans. *Jour. of Bio. 46*: 1-16.
45. Petit C and Sancar A (1999). Nucleotide excision repair: from E. coli to man. *Biochimie*. 81: 15–25.
46. Potts M (2001). Desiccation tolerance: a simple process? *Trends in Microbiol.* 9: 553–559.

47. Raj HD, Duryee FL, Deeney AM, Wang CH, Anderson AW and Elliker PR (1960). Utilization of carbohydrates and amino acids by Micrococcus radiodurans. *Can. Jour. of microbio.* 6(3): 289-298.
48. Rajski SR and Williams RM (1998). DNA cross-linking agents as antitumor drugs. *Chem. Rev.* 98: 2723–2796.
49. Rothkamm K and Lobrich M (2003). Evidence for a lack of DNA double-strand break repair in human cells exposed to very low x-ray doses. Pro. of the Nati. Aca. of Sci. of the U. S. A. 100: 5057-5062.
50. Rouault TA and Klausner RD (1996). Iron-sulfur clusters as biosensors of oxidants and iron. *Trends Biochem. Sci.* 21: 174–177.
51. Sandigursky M, Sandigursky S, Sonati P, Daly MJ and Franklin WA (2004). Multiple uracil-DNA glycosylase activities in Deinococcus radiodurans. DNA Rep. Amst. 3: 163–169.
52. Selby CP and Sancar A (1990). Structure and function of the (A)BC excinuclease of Escherichia coli. *Mutat. Res.* 236: 203–211.
53. Selvam K, Duncan JR, Tanaka M and Battista JR (2013). DdrA, DdrD, and PprA: components of UV and mitomycin C resistance in Deinococcus radiodurans R1. *PLoS One*. 8(7): e69007.
54. Setlow JK and Duggan DE (1964). The resistance of Micrococcus radiodurans to ultraviolet radiation: I. Ultraviolet-induced lesions in the cell's DNA. *Biochim. Et. Biophy. Acta (BBA)*. 87(4): 664-668.
55. Sneath PH, Mair NS, Sharpe ME, Holt JG (1986). Bergey's manual of systematic bacteriology. Volume 2. In: *Williams & Wilkins.*
56. Sukhi SS, Shashidhar R, Kumar SA and Bandekar JR (2009). Radiation resistance of Deinococcus radiodurans R1 with respect to growth phase. *FEMS microbio. let.* 297: 49-53.
57. Sweet DM and Moseley BEB (1976). The resistance of Micrococcus radiodurans to killing and mutation by agents which damage DNA. *Mutat. Res.* 34:175–86.
58. Sweet DM and Moseley BE (1974). Accurate repair of ultraviolet-induced damage in Micrococcus radiodurans. Muta. Res. 23: 311-318.
59. Tanaka M, Narumi I, Funayama T, Kikuchi M, Watanabe H, Matsunaga T, Nikaido O and Yamamoto K (2005). Characterization of pathways dependent on the uvsE,

uvrA1, or uvrA2 gene product for UV resistance in Deinococcus radiodurans. *Jour. of bacterio.* 187(11): 3693-3697.

60. Tanaka M, Earl AM, Howell HA, Park MJ, Eisen JA, Peterson SN and Battista JR (2004). Analysis of Deinococcus radiodurans's transcriptional response to ionizing radiation and desiccation reveals novel proteins that contribute to extreme radioresistance. *Gen.* 168(1): 21-33.
61. Thornley MJ, Horne RW, Glauert AM (1965). The fine structure of Micrococcus radiodurans. *Archiv. für Mikrobiologie*. 51: 267-289.
62. Tian B, Wu Y, Sheng D, Zheng Z, Gao G and Hua Y(2004). Chemiluminescence assay for reactive oxygen species scavenging activities and inhibition on oxidative damage of DNA in Deinococcus radiodurans. *Lumines.* 19(2): 78-84.
63. Tian B, Xu Z, Sun Z, Lin J and Hua Y (2007). Evaluation of the antioxidant effects of carotenoids from Deinococcus radiodurans through targeted mutagenesis, chemiluminescence, and DNA damage analyses. *Biochi. Et. Biophy. Acta.* 1770(6): 902-11.
64. Venkateswaran A, McFarlan SC, Ghosal D, Minton KW, Vasilenko A, Makarova K, Wackett LP and Daly MJ. Physiologic determinants of radiation resistance in Deinococcus radiodurans. *App. enviro. microbio.* 66(6): 2620-6.
65. Work E and Griffiths H (1968). Morphology and chemistry of cell walls ofMicrococcus radiodurans, *J. Bacteriol.* 95: 641-657.
66. Wyman C and Kanaar R (2004). Homologous recombination: down to the wire. *Curr. bio.* 14(15): R629–R631.
67. Xu W, Shen J, Dunn CA, Desai S and Bessman MJ (2001). The Nudix hydrolases of Deinococcus radiodurans. *Mol. Microbiol.* 39: 286–290.
68. Zahradka K, Slade D, Bailone A, Sommer S, Averbeck D, Petranovic M, Lindner AB and Radman M (2006). Reassembly of shattered chromosomes in Deinococcus radiodurans. *Natu.* 443(7111): 569-73.
69. Zhang H, Yin Y, Olman V and Xu Y (2012). Genomic arrangement of regulons in bacterial genomes. *PLoS One.* 7: e29496.

Other techniques

Apart from the project work, following techniques were also learnt:

1. RT- PCR (Reverse Transcription Polymerase Chain Reaction)
2. 2D-PAGE
3. Dose dependent response of *D. radiodurans* gene mutant to DNA damaging stress- Gamma radiation
4. RNA isolation by trizol method
5. Competent cell preparation and transformation in *D. radiodurans*

Annexure

1. **10X TBE (Tris Borate EDTA):**

For 1L of 10X TBE buffer (pH 8.0)

1.	Tris (0.09 M)	108g
2.	Boric Acid (0.088 M)	55g
3.	0.002 M EDTA	0.7g

Dilute 1:10 and use 1X solution

2. **Buffer PA:**

1.	Tris-HCl pH 8.0	50mM
2.	EDTA	10mM
3.	RNase A	100µg/ml

3. **Buffer PB:**

1.	NaOH	200mM
2.	SDS	1%

4. **Buffer PC**:

1.	Gu-HCl	4.2M
2.	Potassium Acetate	0.9M
	Ph	4.8

5. **Wash Buffer-I**

1.	Tris- HCl, pH 7.5	10mM
2.	Ethanol	50%

6. **Wash Buffer-II**

1.	Tris- HCl, pH 7.5	10mM
2.	Ethanol	80%

7. Elution Buffer

1.	Tris- HCl, pH 8.5	50mM
2.	NaCl	50mM
3.	Iso-propanol	15%

8. Competent cells:

8.1 Solution-A

1.	MOPS pH 7.0	418 mg	10 mM
2.	RbCl	214 mg	100 Mm

Dissolved in 200 ml distilled water

8.2 Solution-B

1.	MOPS, pH 6.5	418mg	10mM
2.	$CaCl_2$	147 mg	75mM
3.	RbCl	24.1mg	10Mm

Dissolved in 200 ml distilled water

9. TGY Media

1.	Tryptone	1gm	1%
2.	Glucose	0.1gm	0.1%
3.	Yeast Extract	0.5gm	0.5%

Make up the volume to 100 ml with distilled water.

For TGY agar, add 1.7gm of Agar in 100ml of TGY broth.

10. Tris-HCl 0.5M, pH 6.8

1.	Tris	12.1g
2.	Distilled water	150ml

Adjust pH to 6.8 with 6N HCl and make up the volume to 200 ml with distilled water.

11. Tris-HCl 0.5M, pH 8.8

1.	Tris	36.3g
2.	Distilled water	150ml

Adjust pH to 6.8 with 6N HCl and make up the volume to 200 ml with distilled water.

12. Acrylamide (29.2%), bisacrylamide (0.8%)

1.	Acrylamide	58.4g
2.	Bis acrylamide	1.6g
3.	Distilled water	100ml

Shake well and make up the volume to 200 ml with distilled water. Filter it and store in a dark coloured bottle at 4°C.

13. Tris-Glycine SDS Running Buffer (10X)

1.	Glycine	72g	192mM
2.	SDS	5g	0.1%
3.	Tris	15g	25mM
4.	Distilled water	500ml	

14. Protein gel stain

1.	Methanol	400ml	40%
2.	Acetic Acid	200ml	20%
3.	Coomassie Brilliant Blue (CBB-G250)	2g	0.2%
4.	Distilled water	400ml	

Mix well, filter and store in dark coloured bottle

15. Destaining solution-II

1.	Methanol	100ml	10%
2.	Acetic Acid	100ml	10%
3.	Glycerol	20ml	2%
4.	Distilled water	780ml	

16. 2X SDS Cracking Buffer

1.	SDS	460mg
2.	EDTA	7.6mg
3.	Sodium azide	20mg
4.	Glycerol	2ml
5.	0.1% Bromophenol	200µl
6.	0.5M tris pH 6.8	2.5ml
7.	β-Mercaptoethanol	1ml
8.	PMSF	2mg