Cloning and Characterization of the Single Stranded DNA binding Protein (Ssb) from *Deinococcus radiodurans.*

By

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DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

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List of Publications arising from the thesis

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2. Functional roles of N-terminal and C-terminal domains in the overall activity of a novel single-stranded DNA binding protein of *Deinococcus radiodurans*; Ujaoney A K., Basu B, Muniyappa K. and Apte S K; FEBS OpenBio journal. 2015 (Accepted)

Aman Kumar Ujaoney

DEDICATED TO MY AUNT
LATE Dr. (Ku) JYOTI MAHOBE

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SYNOPSIS

INTRODUCTION

Survival of life forms against acute exposure to DNA damaging stresses is rare. However, the species of orange colored, gram-positive bacterium *Deinococcus* are known to survive such extreme environments. Among the members of the family Deinococcaceae, which comprises of about 47 odd species, some can survive even 15kGy or more of gamma irradiation [1]. *Deinococcus radiodurans* is one of the most studied species which was isolated from irradiated meat cans by Anderson in 1956. It exhibits extremely high resistance to radiation, desiccation and chemical mutagens. Such resistance is achieved by combination of a variety of coping strategies adopted by the bacteria, including (i) robust error-free DNA repair system (ii) Maintenance of high *manganese* to *iron ratios* and (iii) a condensed nucleiod structure [2-4].

Single-stranded DNA binding protein (Ssb) plays an important role in all types of DNA metabolism [5], such as DNA replication, recombination and repair. Ssb protein binds

to single stranded DNA (ssDNA) by electrostatic and base stacking interactions [6-8], through its Oligonucleotide / Oligosaccharide Binding (OB) folds and keeps DNA in single-stranded form, thereby further protecting it from nucleolytic degradation [9]. Ssb protein is also known to regulate activities of several other DNA interacting enzymes/proteins (such as DNA polymerases, primases, RecQ, RecO, RNA polymerases, etc.,) either by direct interaction with them *via* its C-terminal acidic tail, or indirectly by modulating the topology of DNA [5, 10-13].

Ssb protein encoded by *Deinococcus-Thermus* group of bacteria is strikingly different from that of other bacteria. It contains two OB folds and functions as a homodimer [14], in contrast to the homotetrameric prototype *E. coli* Ssb that harbours a single OB fold, or to heterotrimeric eukaryotic RPA (Replication protein A) [10, 15]. Because of its vital housekeeping functions, the deletion of *ssb* gene is lethal. *D. radiodurans* encodes another ssDNA binding protein, the DdrB, but deletion of *ssb* is not complemented by *ddrB* [16].

Biochemical analyses done previously have shown that Deinococcal Ssb binds weakly to ssDNA and displaces a shorter strand of duplex DNA as compared to *E. coli* Ssb. The binding is not significantly affected by salt concentration [17, 18]. Crystal structure of Deinococcal Ssb protein, obtained with and without DNA, has revealed that a structural asymmetry exists between the two OB fold domains, which may have evolved for a specialized role. It also showed that Deinococcal Ssb dimer formation occurs by hydrogen, ionic and van der Waals interactions, using an extensive surface area formed by N-terminal and the connector region. This interaction is very different from L_{45} loop mediated *E. coli* Ssb tetramer formation [7, 19, 20].

Deinococcus radiodurans Ssb polypeptide comprises of 301 amino acids with a molecular mass of 32.6 kDa. The two OB folds are markedly positioned at N and C-terminal region linked by a 10 amino acid long connector region. The C-terminal half of Deinococcal Ssb shares 39% identity and 64% similarity with *E. coli* Ssb protein, while the N-terminal half shares 38% identity and 49% similarity with *E. coli* Ssb [14]. The occluded binding site of the protein is around 50±2 nucleotides, but can form stable complexes with 26-30 nucleotides [18]. Precise role and importance of each OB fold or connector region of Ssb in its activity remains largely unknown.

In this study, a structure-based functional analysis of *Deinococcus radiodurans* Ssb and its regulation was undertaken. Variously truncated variants of Deinococcal Ssb were constructed, over-expressed, purified and biochemically assessed by *in vitro* assays to characterize the likely functions of OB folds and connector region. The expression of Ssb was also studied under various stresses and co-related with the cis-elements present in the *ssb* promoter region. The study has been accomplished with the following objectives:

1. Cloning, over expression of full length *ssb* gene (Ssb_{FL}) as well as truncated Ssb genes, encoding N terminal (with or without connector sequence [Ssb_{NC} & Ssb_{N}]) and C terminal halves (Ssb_{C}) in *E. coli* BL-21 strain and purification of corresponding proteins.

2. Cloning, over-expression and purification of Deinococcal RecA for conducting strand exchange assays with the purified Ssb protein.

3. Biochemical characterization of N (with or without connector sequence) and C terminal halves of Ssb protein, *vis-a-vis* the full length Ssb protein in terms of DNA

binding, topoisomerase assays and trimerization or strand exchange assays with cognate RecA, and

4. Regulation of Deinococcal Ssb protein expression in response to DNA damaging stresses and importance of Radiation Desiccation Response Motif (RDRM) elements in the promoter region.

The work carried out is presented in the thesis as following 4 chapters:

Chapter 1: General introduction

Chapter 2: Materials and Methods

Chapter 3: Results and Discussion

Chapter 4: Summary and Conclusions

Chapter 1: General introduction

This chapter describes general information about *Deinococcus radiodurans*. The information includes classification and microbiological characteristics of *Deinococcus radiodurans* strain R1 and its response to various DNA damaging and other stresses. Different types of responses and pathways adopted by this bacterium to combat severe DNA damage are also described in detail. The already existing information on Deinococcal Ssb protein, its structure, function and crystal structure is reviewed and compared with Ssb proteins from other bacteria. The possible mechanisms underlying regulation of Deinococcal *ssb* gene are summarized, and specific objectives of this study are specified.

Chapter 2: Materials and Methods

This chapter describe the materials, their sources and various experimental techniques used in this study. Details of the microbiological techniques involved, including growth conditions and culture medium of the bacterium and details of stress treatment, including

irradiation, applied in experiments are presented. Various molecular biology techniques including cloning, over-expression and protein purification protocols of various Ssb variants and Deinococcal RecA are specified in detail. Protocols for extraction, quantitation, electrophoretic resolution of proteins, western blotting and immuno detection of candidate proteins are elaborated. DNA binding studies by Electrophoretic Mobility Shift Assay (EMSA) and methods for studying *in vitro* protein interaction by affinity chromatography are specified. Biochemical techniques of topoisomerase assays and strand exchange assays using Deinococcal Ssb variants and cognate Deinococcal RecA as well as non cognate *E. coli* RecA are described in details.

Chapter 3: Results and Discussion

This chapter describes the results obtained from the study and discusses their probable interpretation for the overall function of Deinococcal Ssb protein. The chapter has been subdivided into 3 sections. Each section carries a brief introduction and results obtained from the specific study, followed by a discussion highlighting the implications of the results.

3.1 Cloning, over-expression and purification of Deinococcal full length protein (Ssb_{FL}), N-terminal OB domain (Ssb_N), N-terminal OB domain with connector (Ssb_{NC}), C-terminal OB domain (Ssb_C) and RecA protein, and characterization of various Ssb variants for their oligomeric status, DNA binding affinity, physical and functional interaction.

Ssb_{FL} and Ssb_C were found to exist as dimers, while Ssb_{NC} and Ssb_N proteins displayed polydispersivity, i.e. they formed dimers as well as complex multimers of higher molecular mass which eluted in the void volume of the column. The oligomerization pattern of Ssb

variants clearly demonstrated that the complex multimerization property resided with the N-terminal domain of Ssb, and may help in stabilization of dimeric state of Ssb_{FL}.

The ssDNA binding activity of Deinococcal Ssb variants was evaluated by EMSA, using oligo dT50 over a wide range of protein concentrations. Ssb_{FL} displayed highest ssDNA binding activity (K_D=0.30±0.01 µM) followed by Ssb_C (K_D=0.76±0.04 µM). In comparison, Ssb_N and Ssb_{NC} bound ssDNA weakly and formed different patterns of DNA-protein complexes. Ssb_{NC} displayed a smear-like pattern and a distinct decrease in target DNA with increasing protein concentration, whereas Ssb_N showed couple of discrete bands of DNA-protein complexes, but with no significant decrease in target DNA. The functional interaction between various Deinococcal Ssb variants was evaluated by incubating Ssb_{NC} or Ssb_N with a fixed concentration of Ssb_C, followed by addition of oligo dT50. At a limiting concentration of Ssb_C, no ssDNA-protein complexes were visible. However, as the concentration of Ssb_{NC} or Ssb_N increased (1 – 5 µM), the yield of DNA-protein complexes increased leading to formation of a single ssDNA-protein complex of Ssb_C and Ssb_N and formation of two ssDNA-protein complexes when Ssb_C and Ssb_{NC} interacted. Polydispersive forms of Ssb_N and Ssb_{NC} displayed comparable ssDNA binding capacity, either alone or in association with $Ssb_{C,}$ similar to the unseperated pool of proteins. Deinococcal Ssb_C resembled *E. coli* Ssb, however under *in vitro* conditions Ssb_{NC} or Ssb_N did not enhance ssDNA binding activity of *E. coli* Ssb in EMSA, indicating that $Ssb_{NC/N}$ specifically interact with cognate Ssb_C only.

Direct physical interaction of Ssb_{NC} or Ssb_N with Ssb_C was also ascertained *in vitro*, by affinity chromatography. Ssb_C was first bound to Ni-NTA agarose column through its His-tag and purified Ssb_{NC} or Ssb_N (both without any tag) were then individually passed

through the column. After extensive washing, the column was eluted with 250mM immidazole. Analysis of eluted fractions indicated that Ssb_{NC} or Ssb_N co-eluted with Ssb_C, thereby confirming physical interaction of N-terminal domain of Deinococcal Ssb with its C-terminal domain. The observation that Ssb_{NC} or Ssb_N interact both physically and functionally with Ssb_C suggests that correct folding of the whole complex would result in higher affinity for ssDNA, as compared to Ssb_C alone.

3.2 Analysis of functional roles of various Ssb variants or their combinations in topoisomerase activity and trimerization assays with cognate Deinococcal RecA or non cognate *E. coli* RecA.

Ssb protein is known to enhance the catalytic activity of topoisomerase I and RecA by localized melting and removal of secondary structure of ssDNA intermediate or substrate respectively [21-25]. The effect of Deinococcal Ssb and its variant forms on *E. coli* topoisomerase I activity was studied using M13 double stranded DNA (dsDNA). Topoisomerase I formed a single major band of relaxed DNA in the presence of Ssb_{FL}. But in the presence of Ssb_C, Ssb_{NC} or Ssb_N several intermediate DNA topoisomers were observed. In the presence of N-terminal domains, topoisomerase I generated intermediate topoisomers at lower concentration of Ssb_{NC} or Ssb_N, but exhibited inhibition at higher concentrations of these proteins. In the presence of Ssb_C, topoisomerase I resulted in a higher yield of relaxed DNA at higher concentration than at lower concentration of Ssb_C. The data indicate that both the OB folds of Deinococcal Ssb were independently capable of melting local ssDNA, thereby enhancing topoisomerase I activity. However, Ssb_{NC}/Ssb_N appeared to be inhibitory at higher concentrations and their addition also decreased the positive effect of Ssb_C on topoisomerase I activity. The data suggested that the individual

Ssb domains may support the activity of native topoisomerases during DNA damage repair in the recovery phase.

Ssb plays a very important role in promoting strand exchange assay by removing secondary structures in ssDNA. The ability of Deinococcal Ssb variants to stimulate DNA strand exchange promoted by the cognate RecA or non cognate *E. coli* RecA was therefore assessed. Addition of Ssb_{FL} up to 3 µM increased the efficiency of strand exchange as the yield of nicked circular DNA product increased. However, addition of Ssb_C, Ssb_N or Ssb_{NC} could not form the product molecules. *In vitro,* combination of Ssb_N or Ssb_{NC} with Ssb_C also did not augment product formation. Competitive strand exchange assays carried out with Ssb_{FL} in combination with all other variants showed that Ssb_C, Ssb_{NC} or Ssb_N did not inhibit the strand exchange reaction promoted by Ssb_{FL}. EMSA carried out under strand exchange assay conditions showed that the ssDNA-protein complexes (Ssb_{NC}/Ssb_N with Ssb_C) were indeed present, but did not assist in strand exchange. Thus, the N-terminal and C-terminal Ssb variants did form expected DNA-protein complexes under strand exchange assay condition but were unable to remove secondary structures of virion ØX174 ssDNA. Ssb_{FL}, which contains naturally linked N-terminal and C-terminal portions by a connector region, not only bound to ssDNA with highest affinity compared to other variants of Ssb but could also remove secondary structures, thereby enhancing RecA activity.

3.3 Effect of various DNA damaging stresses on *in vivo* Ssb protein expression.

This study was carried out by exposing *D. radiodurans* cells individually to gamma rays, mitomycin C, hydrogen peroxide, UV rays or desiccation. When exposed to gamma rays (7 kGy) or mitomycin C (20 µg per ml, for 15 min), *D. radiodurans* cells showed significant time-dependant induction of Ssb levels, as visualized by immuno-

detection during post stress recovery. Both these stresses inflict double strand breaks and adducts formation, which triggers transcriptional induction of the *ssb* gene. A distinct radiation dose-dependent increase in Ssb levels was also observed during post irradiation recovery (PIR), but was abolished when rifampin was added during PIR. Exposure to UV rays or hydrogen peroxide, which mainly forms to pyrimidine dimers, or cause individual base damage or single strand breaks, did not alter the cellular Ssb levels. Surprisingly, 1 - 6 weeks of desiccation also did not affect Ssb levels possibly due to lower magnitude of DNA damage caused in comparison to that by acute radiation exposure. Radiation induced transcriptional activation of *ssb* gene was shown to be controlled by two radiation and desiccation response motifs (RDRM), which seemed to work additively.

Chapter 4: Summary and Conclusion

Deinococcus-Thermus group of bacteria, known to thrive in extreme environments which readily cause breach in DNA integrity, encode a novel Ssb composed of 2 asymmetric OB folds linked by a 10 amino acid long connector. The elevated Ssb protein levels following radiation and mitomycin C stress suggest that disruption of DNA integrity elevates transcriptional levels mediated by RDRM elements upstream of the *ssb* gene. Ssb plays the important role of keeping DNA in single stranded conformation for repair, simultaneously protecting it from nuclease digestion. Bioinformatic analyses together with crystal structure data have earlier implicated C-terminal OB fold in ssDNA binding [19] with only a few residues of N-terminal OB fold also taking part in ssDNA binding [7]. The data obtained in this study clearly demonstrate the functional significance of the individual OB fold of the deinococcal Ssb protein. The ssDNA binding capability primarily resides in the C-terminal

OB fold and is aided further by the N-terminal OB fold which is mainly engaged in multimerisation. N-terminal OB fold together with C-terminal OB fold, displays improved ssDNA binding *in vitro*. However, specific interactions and cooperative contribution of both the OB folds in Ssb_{FL} are necessary for efficient melting of secondary structures in ssDNA, a feature essential for DNA replication, recombination and repair. Evolution of such atypical Ssb appears to be prompted by a necessity to protect as much ssDNA templates as possible for strand annealing followed by homologous recombination repair, following gamma irradiation induced massive DNA damage. Cooperative interactions between the multimerization inducing N-terminal OB fold and the ssDNA binding C-terminal OB fold appear to functionally complement each other to accomplish rapid nucleation and protection of ssDNA templates, to be used for very efficient and error-free DNA repair in Deinococci.

References

1. de Groot A, Chapon V, Servant P, Christen R, Saux MF, et al. **2005**. *Int J Syst Evol Microbiol* 55: 2441-6
2. Zahradka K, Slade D, Bailone A, Sommer S, Averbeck D, et al. **2006**. *Nature* 443: 569-73
3. Daly MJ, Gaidamakova EK, Matrosova VY, Vasilenko A, Zhai M, et al. **2004**. *Science* 306: 1025-8
4. Levin-Zaidman S, Englander J, Shimoni E, Sharma AK, Minton KW, Minsky A. **2003**. *Science* 299: 254-6
5. Shereda RD, Kozlov AG, Lohman TM, Cox MM, Keck JL. **2008**. *Crit Rev Biochem Mol Biol* 43: 289-318
6. Casas-Finet JR, Khamis MI, Maki AH, Chase JW. **1987**. *FEBS Lett* 220: 347-52
7. George NP, Ngo KV, Chitteni-Pattu S, Norais CA, Battista JR, et al. **2012**. *J Biol Chem* 287: 22123-32
8. Overman LB, Lohman TM. **1994**. *J Mol Biol* 236: 165-78
9. Slade D, Radman M. **2011**. *Microbiol Mol Biol Rev* 75: 133-91
10. Meyer RR, Laine PS. **1990**. *Microbiol Rev* 54: 342-80
11. Furukohri A, Nishikawa Y, Akiyama MT, Maki H. **2012**. *Nucleic Acids Res* 40: 6039-48
12. Reddy MS, Guhan N, Muniyappa K. **2001**. *J Biol Chem* 276: 45959-68
13. Antony E, Weiland E, Yuan Q, Manhart CM, Nguyen B, et al. **2013**. *J Mol Biol* 425: 4802-19
14. Eggington JM, Haruta N, Wood EA, Cox MM. **2004**. *BMC Microbiol* 4: 2
15. Wold MS. **1997**. *Annu Rev Biochem* 66: 61-92
16. Lockhart JS, DeVeaux LC. **2013**. *PLoS One* 8: e71651
17. Kozlov AG, Eggington JM, Cox MM, Lohman TM. **2010**. *Biochemistry* 49: 8266-75
18. Eggington JM, Kozlov AG, Cox MM, Lohman TM. **2006**. *Biochemistry* 45: 14490-502
19. Bernstein DA, Eggington JM, Killoran MP, Misic AM, Cox MM, Keck JL. **2004**. *Proc Natl Acad Sci U S A* 101: 8575-80
20. Raghunathan S, Kozlov AG, Lohman TM, Waksman G. **2000**. *Nat Struct Biol* 7: 648-52
21. Muniyappa K, Shaner SL, Tsang SS, Radding CM. **1984**. *Proc Natl Acad Sci U S A* 81: 2757-61
22. Kowalczykowski SC, Krupp RA. **1987**. *J Mol Biol* 193: 97-113
23. Ngo KV, Molzberger ET, Chitteni-Pattu S, Cox MM. **2013**. *J Biol Chem* 288: 21351-66
24. Jain S, Zweig M, Peeters E, Siewering K, Hackett KT, et al. **2012**. *PLoS One* 7: e35285
25. Sikder D, Unniraman S, Bhaduri T, Nagaraja V. **2001**. *J Mol Biol* 306: 669-79

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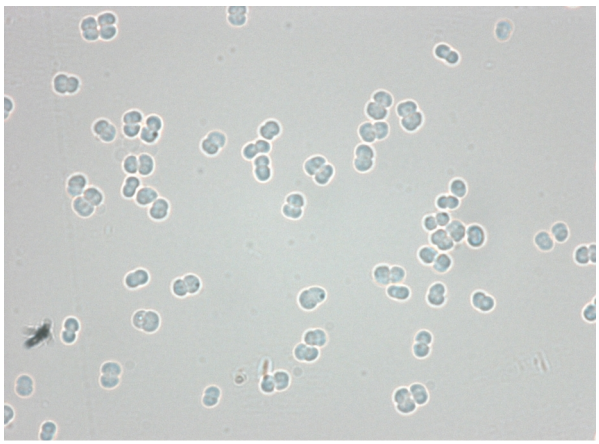
Chapter 1
Introduction

Deinococcus radiodurans is Gram positive, red pigmented, non sporulating and non-pathogenic bacterium. The name is derived from the greek word meaning strange grain or berry. *Deinococcus* generally exist as smooth convex colonies in diads and tetrads with the diameter ranging from 0.3 µm to 3.5 µm [26]. The bacterium is a mesophile, grown preferentially in protein rich medium of tryptone and yeast extract optimally at 32°C with aeration [27]. When grown in 1% tryptone, 0.1% glucose and 0.5% yeast extract (TGY), the cell doubling time is 90 min and colonies take 2 days to be clearly seen, on TGY supplemented agar plates. Recombinant Deinococcal cells have been shown to have potential application in bioremediation process [28, 29]. The appearance of *Deinococcus radiodurans* strain RI under light microscopy and electron microscopy is shown in Fig 1.1

(A) (B)



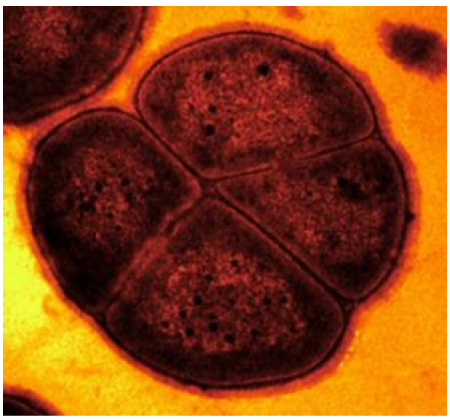


Fig. 1.1. Photograph of *Deinococcal radiodurans*. (A) Photomicrograph of a laboratory culture of *Deinococcus radiodurans* strain (R1) taken at 1000X magnification. (B) Electron micrograph of Deinococcal cells (Source: http://bioweb.uwlax.edu).

1.1. Classification and evolution of *Deinococcus radiodurans*.

The genus *Deinococcus* comprises over 47 isolated species distinguished on the basis of their ability to survive DNA damaging stresses including radiation stress [30] and desiccation stress [31], inhabiting diverse environment such as hot springs, animal gut, Antarctic environment and deserts [32]. *Deinococcus radiodurans* strain R1 was first isolated from a irradiated meat can by Anderson [33] and was classified as *Micrococcus radiodurans* on the basis of morphological and physiological characteristics. However after 16S rRNA sequencing analysis, the bacterium was included in a new family of *Deinococcaaceae* as it formed a unique phylogenetic group and was named as *Deinococcus radiodurans* [34]. The classification is as follows:

Table 1.1. Classification of *Deinococcus radiodurans*

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

The phylogenetic tree based on Clusters of Orthologous Group of proteins (COGs) made on the basis of proteins encoded by bacterial genome indicated close relationship between *Thermus thermophilus* and *Deinococcus radiodurans* genomes, with the single megaplasmid of *Thermus* and the DR177 megaplasmid of *Deinococcus* being probably inherited from a common ancestor. *Themus* and *Deinococcus* lineages seem to diverge in evolution. *Thermus* group of bacteria evolved by extensive gene losses, but acquired many new genes from thermophiles for its survival in extreme environment. *Deinococcus*, in contrast did not lose much of the gene repertoire in evolution but acquired other (10-15%) genes by horizontal gene transfer [35], for its survival in a variety of extreme environmental conditions [36]. The

presence of archaebacteria close to Deinococcus/ Thermus group signifies their closeness in the evolutionary process (Fig 1.2).

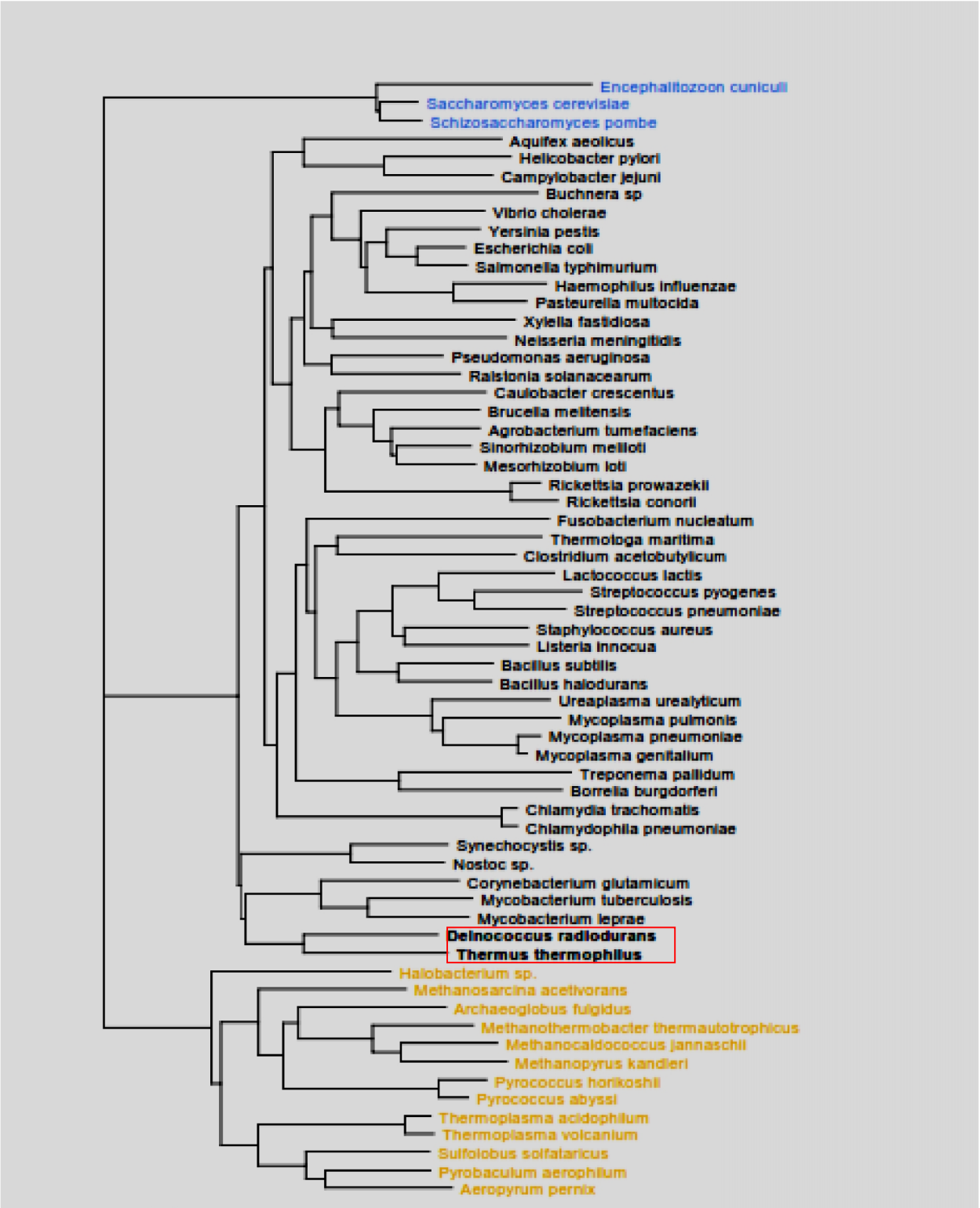


Fig. 1.2. Phylogenetic tree based on COGs. *Deinococcus/ Thermus* clade is marked in bold in red box. Black color stands for bacteria, yellow for archaea and blue for eukaryotes. (Source: Omelchenko, M. V et. al, 2005 [36]).

Trupera radiovictrix is another radioresistant bacterium and most alkaliphilic organism of *Deinococcus* lineage which grows at optimum temperature of 50°C [37]. Apart from *Deinococcus* lineage, there are many other bacteria which are radiation resistant. Examples include mostly gram positive bacteria such as *Rubrobacter xylanophilus*, *Rubrobacter radiotolerans*, *Lactobacillus plantarum* and *Kineococcus radiotolerans*. The list is not comprehensive and also includes some archaebacteria and eukaryotes comprising of green algae, slime mold and fungi [9].

1.2. Cellular architecture of *Deinococcus radiodurans*.

Deinococcus radiodurans is a Gram-positive bacterium, with more complex cell wall architecture than other gram positive bacteria. It comprises of 5 layers. (I) the cytoplasmic membrane, (II) the peptidoglycan containing holey layer composed of mucopeptide, (III) compartmentalized layer, (IV) the interior layer and (V) the fragile soft layer containing hexagonally packed S-layer composed of lipids, carotenoids, proteins and polysaccharides [38, 39]. Alkylamines present in phosphoglycolipids of membrane lipids are unique to *Deinococci* [40]. Unsaturated fatty acids present in lipoproteins contribute to fluidity of membrane, which play important role in membrane dynamics during desiccation stress. Lipoproteins comprise of even and odd saturated fatty acids [41, 42]. During growth of bacterium, the cells divide in two planes with septa originating from opposite planes, perpendicular to the previous set [43]. Interestingly, X-ray fluorescence microprobe analysis has shown that Fe^{2+} ions get accumulated at the dividing septum while Mn^{2+} ions get concentrated at nucleiod during cell division [44].

1.3. *Deinococcus radiodurans* metabolism.

Deinococcus radiodurans is an organotrophic bacterium, with its energy requirement mainly met by proteolysis. Tryptone and yeast extract present in rich medium mainly contribute to amino acids and peptides, while glucose contributes only marginally to its energy requirement [27, 45]. When grown on skimmed milk plates it forms a large halo indicative of its high proteolytic activity [46]. Such metabolic behavior helps *Deinococcus* in controlling its oxidative stress in following ways:

1. The proteolytic lifestyle of bacterium is aided by 10 secreted subtilisin – like proteases and ABC transporters of peptides and amino acids helping in protein recycling [46, 47], which decreases new demand for bio-synthesis and contributes antioxidant complexes of amino acid and peptides with manganese [48].

2. Deinococcal group of bacteria assimilate glucose through glycolysis, gluconeogenesis, pentose phosphate pathway (PPP), the tricarboxylic acid cycle and glyoxalate bypass [35]. PPP pathway converts glucose to precursors of deoxynucleoside triphosphate (ribose-5-phoshate, glyceraldehye -3-phosphate and NADPH) and is very active in *Deinococcus* in comparison to *E. coli*. These intermediates are further used in nucleic acid production as well as cofactors for antioxidant systems [9, 49]. PPP normally incorporates 8% of ^{14}C from glucose in DNA [45]. It is also upregulated following irradiation [50]. In response to stress, *Deinococcus* upregulates glyoxylate bypass, which reduces the concentration of $FADH_2$ and NADH [46, 47] by directly converting isocitrate into succinate and glyoxylate. This process ultimately reduces production of endogenous reactive oxygen species (ROS) because of decreased interaction of electrons from $FADH_2$ and NADH with oxygen, leading to less production of superoxide radical and hydrogen peroxide [51]. Endogenous ROS production is

inherently less in Deinococcal group of bacteria, as against other radiosensitive bacteria, because of less (56% fewer) respiratory chain enzymes and 65% fewer iron-sulphur cluster enzymes [46]. The decreased concentration of iron containing enzymes also decreases presence of free iron in cytoplasm, upon exposure to various stresses and ultimately reduces hydroxyl radical ($OH^{\cdot}$) production by Fenton-type reaction [51]. Thus, by providing precursors for nucleic acid production and generating energy by altered Kreb cycle, glucose prevents formation of ROS and, in turn, partially contributes to radioresistance of *Deinococci*.

3. Lastly, but importantly, the presence of polyphosphate bodies in the cytoplasm acts as a reserved source of energy for various metabolic processes involved in ATP synthesis. They also act as phosphorylating agent for proteins, sugars, nucleosides and more importantly form manganese complex with intracellular manganese ions which ultimately act as scavenger of ROS [48, 52-54].

Deinococcal metabolome has thus evolved to contribute to extreme resistance to DNA and non-DNA damaging stresses by reducing indigenous production of ROS as well as scavenging free radicals generated from external ROS stress by combing cellular metabolites with manganese ions [9].

1.4. Genome architecture of *Deinococcus radiodurans*.

Deinococcus genome consists of 3.28 mega bases distributed into two chromosomes and two plasmids. The chromosome I consists of 2,648,638 base pairs, while chromosome II consists of 412,348 base pairs. Megaplasmid I contains 177,466 base pairs and the smaller plasmid II contains 45,704 base pairs. Many horizontally acquired genes from other organism are present on the megaplasmid. The natural competence of this organism further aids in horizontal gene

transfer [36]. The genome encodes about 3,187 open reading frames (ORF) and has a high GC content of 66.6% [55, 56]. Deinococcal cells contains 4-10 copies of genome in exponentially growing cells, the number of copies keep on changing according to growth stage and medium condition [57, 58]. The multiplicity of genome copy number does contribute to resistance against DNA damaging stresses by providing ample targets for homologous recombination based repair during recovery. However, till now there is no direct evidence linking genome copy number with radiation resistance.

1.5. DNA repair in *Deinococcus radiodurans* in response to stress.

DNA damage can be caused by a variety of stresses. Gamma irradiation or desiccation causes generation of double strand breaks (DSB) which are almost 10 times less than single strand breaks (SSB) and base damage. Mitomycin-C causes DNA interstrand cross-links which leads to formation of adducts and subsequently to double strand breaks upon replication, repair or recombination. UV rays produce pyrimidine dimers and exposure to methyl methane sulfonate (MMS), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), nitrous acid, hydroxylamine or hydrogen peroxide causes base and nucleotide damage. *Deinococcus radiodurans* is 30 fold more resistant that *E. coli* and 1000 fold more resistant than humans, against radiation stress and can repair almost 200 DSBs and 190 cross-links per genome copy without any loss of viability [30, 57, 59].

The extreme radioresistance shown by *Deinococcus* species can be attributed largely to its immense potential to protect itself against oxidative stresses, since the common DNA repair mechanisms found in this bacterium are similar to, or rather more simpler than that in, other radiosensitive bacterial species of *E. coli* or *Shewanella oneidensis* [35, 48, 60, 61].

The known mechanisms underlying tolerance to different types of DNA damaging stresses in *Deinococcus* are briefly discussed below:

1.5.1. *Ionizing radiation resistance.*

Deinococcus radiodurans is extremely resistant to ionizing radiation. Typical dose response curve of *Deinococcus radiodurans, E. coli* and *Thermus thermophilus* for γ-rays is shown in Fig 1.3 [36].

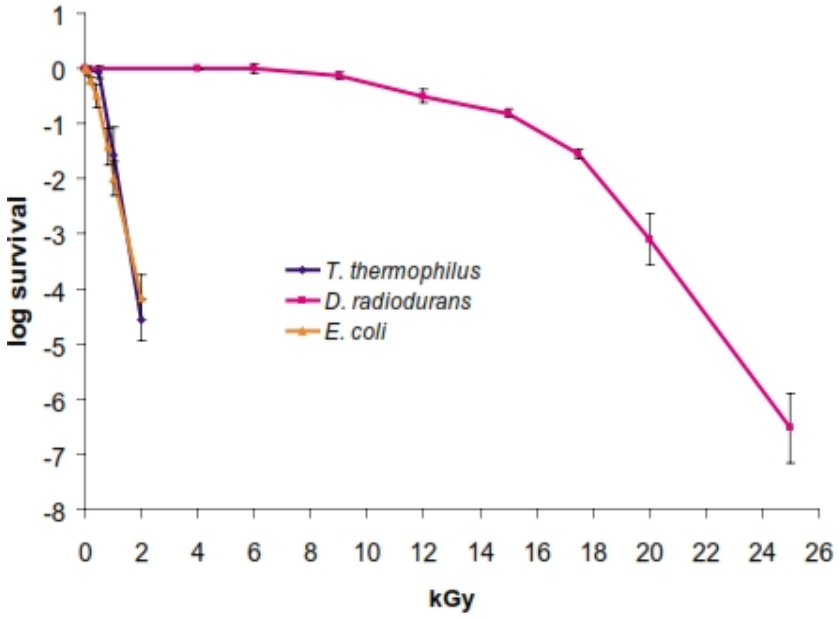


Fig. 1.3. Radiation dose response curve of *Deinococcus radiodurans, E. coli* and *Thermus thermophilus*. *D. radiodurans* survival is marked in magenta, *E. coli* survival is marked in yellow and *T. thermophilus* survival is marked in indigo. (Source: Omelchenko, M. V et. al, 2005 [36]).

The interaction of ionizing radiation with any biomolecule (DNA, proteins, lipids etc.) is of two types (1) direct interaction, and (2) indirect interaction by generating highly reactive free radicals, with hydroxyl radical being the most reactive and damaging species (Standard reduction potential 2.31V) [49, 51]. Typically, during irradiation stress, 20% of DNA damage is caused by direct interaction of γ-photons with DNA while ~80% of DNA damage is caused

by indirect interaction with reactive oxygen species (ROS) [49]. In case of DNA, exposure to ionizing radiation leads to multiple types of DNA damage namely, double strand breaks (DSBs), single strand breaks (SSB), individual base damage and inter-strand cross links [62]. A dose of 6 kGy of γ-rays typically induces approximately 200 DSBs, over 3000 SSBs and many individual base damages. DSBs formed in Deinococcal genome are a result of "single event" as opposed to "double event", where two SSBs are formed in close proximity resulting in DSBs [63, 64]. The radiation resistant and sensitive species mostly differ in the ratio of oxidative DNA damage rather than DNA damage occurring due to direct interaction of ionizing radiation [65]. The strand break repair in *Deinococcus* follows biphasic kinetics with rapid initial step where SSBs are repaired followed by a slow step where DSBs are repaired [66-68]. SSBs generated due to radiation effects are mostly repaired by polynucleotide kinases and DNA ligases [69]. The DSBs generated due to irradiation are repaired in three steps (1) DNA degradation process with little repair, (2) rejoining of DNA fragments in parallel with extensive polymerase activity, and (3) the resumption of growth upon completion of all DNA repair. The DSBs are repaired by two RecA mediated homologous recombination processes (1) ESDSA (extended synthesis dependant strand annealing, and (2) homologous recombination at crossovers [2, 9, 70]. The mechanism of ESDSA (Fig. 1.4) requires extensive polymerase activity at the start of the process which resulted in its discovery [2]. Other proteins which substantially contribute to this process are UvrD and RecJ, which processes DSBs into 3' single stranded substrates. Ssb protein plays a major role by coating 3' single stranded substrates, thus protecting it. The RecF protein then binds to SS-DS junction, which mediates assembly of RecOR complex. RecOR ultimately displaces Ssb protein and loads RecA at 3' end for it to prime DNA repair synthesis. Following RecA/RadA

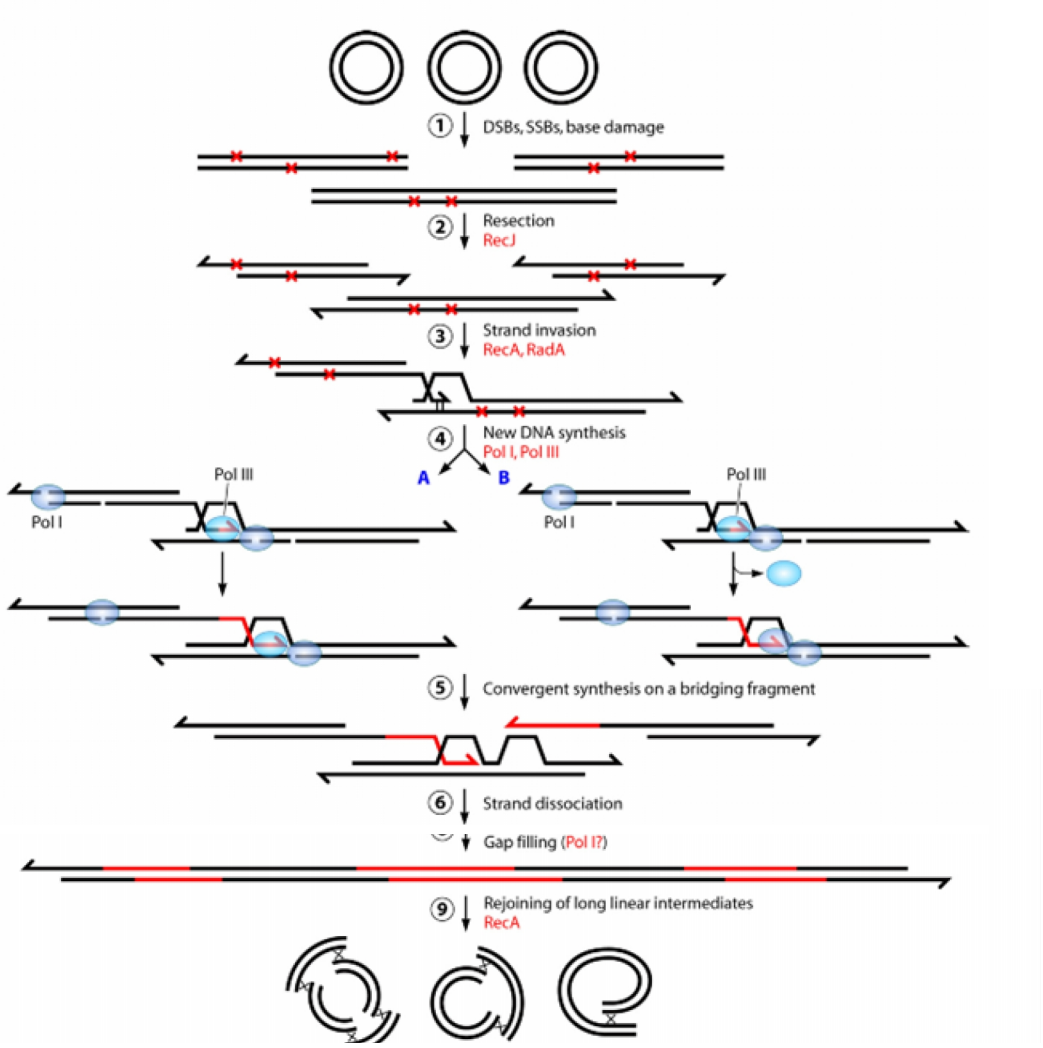


Fig. 1.4. ESDSA pathway in *Deinoccocus radiodurans*. Major steps associated with ESDSA pathway are shown. (Source: Slade and Radman, 2011 [9]).

mediated priming, DNA repair synthesis is mediated by DNA polymerase I and/or III. The unwinding of dsDNA upon D-loop formation is mainly mediated by variety of helicases (RuvAB, RecD, RecQ etc.). DNA helicases progressively dissociates the newly synthesized strands from D-loops which then re-anneal with its complementary partner. The flaps present

in these strands are removed by action of SbcCD. The mature circular chromosomes are then synthesized from these ESDSA linear fragments by crossover events mediated by RecA protein at overlapping homology [9]. Ssb protein plays a very important role in RecA mediated processes.

Oxidized bases are repaired by base excision repair system (BER)**,** which comprises of AP endonuclease and uracil glycosylase [71]. The mismatch repair system comprises of MutS1, MutL and UvrD which also enhances the fidelity of replication and recombination processes [72]. The genome condensation mediated by ring like nucleiod morphology or by structural proteins like HU and DPS family of proteins also help in minimizing damage following radiation as they prevent diffusion of DNA fragments as well as reduce accessibility of DNA fragments to various nuclease and to some extent prevent the effect of free radicals generated from water on DNA fragments [4, 73, 74].

The radiation induced oxidative stress can be clearly observed in case of proteins. The free radicals generated due to radiolysis of water carry out a series of different changes in various amino acids, which are important constituents of proteins. Some of these are, (1) carbonylation of proline, lysine, arginine and threonine (2) oxidation of sulphydryl group of cysteine and methionine and (3) introduction of hydroxyl group in ring chained amino acids of phenylalanine and tryptophan and (4) breakage of peptide bonds [75].

In *Deinococcus radiodurans* the oxidative stress tolerance is mainly achieved by two types of ROS scavengers, non-enzymatic soluble manganese complexes [44, 48, 61] and by enzymes (superoxide dis mutase, catalase and peroxidases). *Deinococcus radiodurans* cells maintain high levels of intracellular manganese levels (0.2-0.4mM) [3, 76] as well as a high Mn^{2+} to Fe^{2+} ratio of 0.24 [3] . It accumulates close to 150 times more Mn^{2+} than radiosensitive

bacteria such as *S. oneidensis*. On the other hand *S. oneidensis* accumulates 3.3 times more Fe^{2+} than *Deinococcus radiodurans* resulting in its radio sensitivity [46]. The high Mn/Fe ratio minimizes radiolysis of water inside the cells and contributes significantly to extreme radiation and desiccation tolerance. The relationship between Mn to Fe ratio of various organisms and their survival to gamma rays is shown in Fig 1.5 [77].

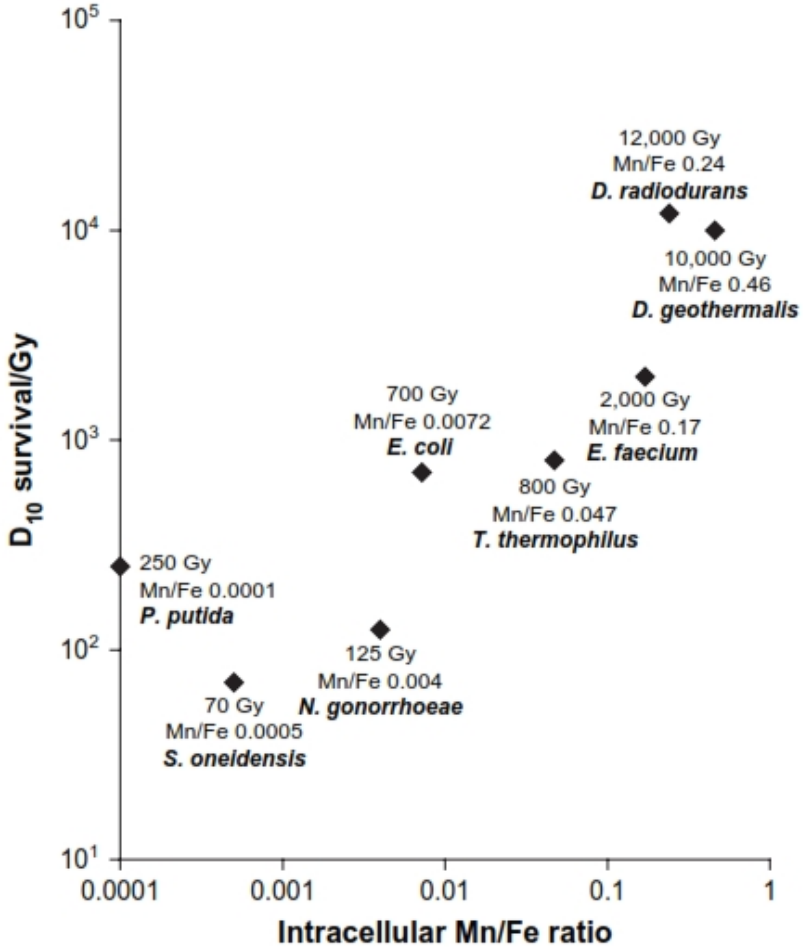


Fig. 1.5. D_{10} survival values and intracellular Mn/Fe content of various organisms. (Source: Daly, M.J, 2006 [77]).

The chromosome in Deinococcal cells also preferentially binds Mn(II), which can mimic catalase and SOD like activities, thus preventing damage by avoidance of Fenton-type chemistry. Among the ROS scavenges proteins, *Deinococcus* encodes 3 catalases, four superoxide dismutases (SOD) and two peroxidases. SODs convert superoxide ions into hydrogen per-oxide; this hydrogen peroxide is subsequently converted to water and oxygen by catalase enzyme activity. Catalase activity has been reported to be 15 fold higher in *Deinococcus* than in *E. coli* [78]. It was also reported that catalase activity is positively regulated by transcriptional regulator DrRRA [79] and negatively by OxyR [80]. The Mn-dependent SOD is constitutively expressed in Deinococcus and is believed to be more efficient than its counterpart in *E. coli* [81], although the structure of Mn-SOD is almost identical in both the bacterium [82]. The Deinococcal mutants in SOD and catalase were found to be sensitive to oxidizing agents and also to high doses of γ-radiation than wild type bacterium [83].

Deinococcal cells also encode only 10 predicted cytrochromes and 39 flavoproteins (which are rich source of free Fe^{2+} ions under stress conditions) in comparison to 53 cytrochromes and 59 flavoproteins encoded by bacteria such as *S. oneidensis* [46]. Carotenoids present in *Deinococcus radiodurans* also contribute importantly to ROS scavenging. Deinoxanthin specially shows a strong ability to scavenge hydrogen peroxide and singlet oxygen species, thus protecting DNA from reactive oxygen species [84]. Deletion mutants of carotenoids biosynthetic pathway were found to be colorless and more sensitive to radiation, hydrogen peroxide and UV stress [85], although some pigment less mutants of *Deinococcus radiodurans* and *Deinococcus radiopugnans* are as radioresistant as wild type [55, 86].

Among other antioxidants, pyrroloquinoline-quinone (PQQ) coenzyme present in *Deinococcus* can scavenge different kinds of ROS, especially OH˙. When expressed in *E. coli* the corresponding *pqqE* gene resulted in efficient scavenging of ROS and protection of proteins against carbonylation [87] It also stimulated catalase and SOD activity [88].

1.5.2. *Desiccation tolerance*.

Deinococcus radiodurans is severely desiccation tolerant with LD_{50} dose of close to 2 years [9]. Typical survival curve of *Deinococcus radiodurans*, *E. coli* and *Thermus thermophilus* following desiccation stress is depicted in Fig 1.6 [36].

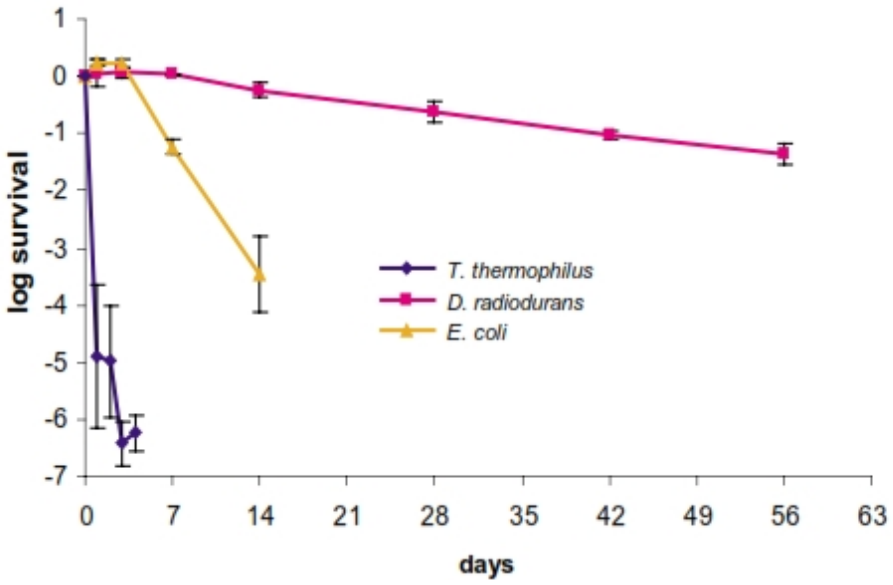


Fig. 1.6. Desiccation stress response curve of *Deinococcus radiodurans*, *E. coli* and *Thermus thermophilus*. *D. radiodurans* survival is marked in pink, *E. coli* survival is marked in yellow and *T. thermophilus* survival is marked in indigo. (Source: Omelchenko, M. V et. al, 2005 [36]).

Desiccation is a severe form of stress which mainly leads to protein denaturation and formation of ROS that ultimately causes oxidative damage to lipids, proteins and DNA [89, 90]. Six weeks of desiccation leads to 60 DSBs in *Deinococcus* genome [91]. The DNA repair and oxidative stress tolerance during desiccation is taken care by various DNA repair proteins and non enzymatic soluble manganese complexes as well as enzymatic activity of catalases, SODs and peroxidases as discussed above. *Deinococcus* group is also unique in having homologues of plant desiccation associated proteins (LEA protein family) [35]. The importance of this group of proteins was seen in *Deinococcus deserti*, where they were easily detected and thus predicted to help in adaptation of bacteria to the desert condition [92]. Desiccation studies with radiosensitive mutants of *Deinococcus* have revealed that adaptation to radiation stress may be incidental and probably a consequence of adaptation to desiccation stress [91]. This is based on the fact that radiation sensitive mutants of *Deinococcus radiodurans* were found to be desiccation sensitive as well and desiccated (air dried) cells show greater resistance to irradiation and UV stress than cells in solution [93].

1.5.3. *UV resistance*.

Photoreactivation, common to all bacteria is totally absent in *Deinococcus* [94]. Exposure to UVC (100 to 295nm) radiation leads to formation of cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4)-pryrimidone photoproducts (6-4 PPs) [95, 96]. The repair of UV associated lesions occurs in 2 steps, (1) excision repair and (2) recombinational repair. In *Deinococcus radiodurans* the excision repair for removal of pyrimidine dimers is carried out by 2 pathways namely UvrABC excision repair pathway and UV damage endonuclease (UVDE) pathway [94, 96]. UvrABC repair proteins (UVR ABC exinuclease) recognize the

structural damage on UV irradiated DNA and create dual incision at the damage site for the removal of damaged base [97]. The UVDE pathway on the other hand is mediated by endonuclease β which introduces a single nick at 5' position to the lesion, following which other repair proteins repair the lesion [98]. UVDE pathway efficiently removes both CPDs and 6-4PPs efficiently, while UvrABC is specific for 6-4PPs. UvrABC system is considered more important than UVDE pathway [99]. The replication fork collapse created at the site of UV induced nick or at single stranded gap (due to base excision by exinuclease) is resolved by the normal recombination process [100, 101].

1.5.4. *Mitomycin-C resistance*.

Mitomycin-C treatment leads to alkylation of 2 guanine residues at N^2 positions leading to formation of bisguanine adducts [102]. Such cross-links block replication and transcription and lead to more DSBs after the associated enzymes try to carry out the replication, repair or transcription [103, 104]**.** The DSBs formed due to mitomycin-C are repaired by nucleotide excision repair pathway and RecA mediated recombination repair pathway [59, 97, 105].

The recombination repair is the core mechanism of repairing major DNA lesions such as DSBs. The enzymes which play important roles in such repair are discussed below:

1.6. Major enzymes involved in recombination repair

1.6.1. *The RecFOR protein complex.*

Repair of DNA by recombination is required in nearly all types of DNA damaging stresses. The RecA protein is the central workhorse protein of this pathway. RecA mediated

recombination is a ATP driven process and requires either a RecBCD or a RecFOR protein complex for processing and loading of RecA onto DNA. In *E. coli* the processing of DNA molecules and loading of RecA is mainly carried out by RecBCD pathway, where RecBCD heterotrimer acts as helicase as well as an exo- and endonuclease [106, 107]. *Deinococcus radiodurans* does not have RecB and RecC homologues and as such the nuclease activity in Deinococcal cell free extracts is quite low [108]. *In trans* overexpression of *E. coli* RecBC or SbcB (3'-5' exonuclease) in *D. radiodurans* makes recombinant *Deinococcus* cells radiosensitive due to interference in DNA repair process [108, 109].

The recombination repair in Deinococcal cells is mediated by RecFOR pathway. *Deinococcus radiodurans* has all the components of RecFOR pathway namely RecF, RecO, RecR, RecJ and UvrD helicase. In RecFOR pathway the UvrD helicase unwinds the DNA, while RecJ digests the 5' end. The 3' tailed single strands generated, are coated with Ssb molecules. Then RecF binds ssDNA-dsDNA junction and promotes the assembly of RecOR complex onto the junction. RecOR displaces Ssb and loads RecA onto the 3'end to complete the recombination process [9]. These proteins are required for normal growth, recombination and repair. Deletion (Δ*recFOR)* mutants of *D. radiodurans* are extremely radiosensitive and show slow assembly of chromosomal fragments with no DNA synthesis and reduced DNA degradation [110] .

1.6.2. *The RecA protein*.

RecA protein is involved in homologous recombination during normal growth or during DNA repair in bacteria. The nucleoprotein aligns bound ssDNA with linear dsDNA and carries out ATP dependent strand exchange among them. The *rec*A mutation is lethal. Conditional lethal *recA* mutants show recessed growth and radiation sensitivity [59, 111]. Transcriptome and

proteomics studies have shown LexA independent induction [112, 113] in levels of RecA following irradiation [47, 112]. The transcriptional regulator DrRRA positively controls *rec*A expression in non-stress conditions, while global regulators DdrO and PprI may be involved in induction of *rec*A gene following stress exposure [60, 79]. Deinococcal RecA protein is unique in carrying out inverse strand exchange with preference for dsDNA in comparison to ssDNA substrate, thus implying its importance during massive DNA damage where probability of finding linear dsDNA is more [114].

1.6.3. *The Ssb protein*.

Ssb protein is involved in all facets of DNA metabolism, be it replication, repair or recombination. All aspects of DNA metabolism require opening of dsDNA to single stranded DNA intermediates so that the genetic information can be processed accordingly. The single stranded DNA intermediates are prone to chemical or nucleolytic attack, those are difficult to resolve, and may lead to major loss to genome architecture. To avoid such a situation single stranded DNA binding proteins have been evolved as important housekeeping proteins [115], which bind ssDNA with high affinity in sequence-independent manner [116] and protect ssDNA from degradation. Ssb protein as such is quite resistant to heat denaturation, alkaline degradation and resistant to treatment with guanidine hydrochloride [117].

Ssb protein binds to ssDNA substrate through its OB folds using a combination of electrostatic and base stacking interaction with phosphodiester backbone and nucleotide bases of substrate [8, 20].

Ssb proteins have oligomerization property, which leads to multiplicity in the number of OB folds interacting with ssDNA simultaneously [118, 119]. The protypical eubacterial Ssb

protein studied in great details is of *E. coli*, which exists as a tetramer in solution with its 4 OB folds available for binding to ssDNA depending on solution condition [117, 120]. *E. coli* Ssb binds to ssDNA in two binding modes of $(SSB)_{35}$ and $(SSB)_{65}$. In $(SSB)_{35}$ mode, approximately 35 nucleotides of ssDNA substrate interact with 2 subunits of Ssb. This mode has unlimited co-operativity and appears as long protein clusters along ssDNA [121]. $(SSB)_{35}$ is preferred under low salt, high Ssb to ssDNA ratios and is proposed to function in DNA replication [8, 121]. In $(SSB)_{65}$ mode, approximately 65 nucleotides of ssDNA substrate interact with all 4 subunits of Ssb. This mode has limited co-operativity and shows little tendency to form protein clusters along ssDNA [122]. $(SSB)_{65}$ is preferred under high salt, low Ssb to ssDNA ratios and is proposed to function in DNA recombination mediated by RecA [121, 123]. The tryptophan fluorescence quenching was higher in $(SSB)_{65}$ mode than in $(SSB)_{35}$ mode [120].

The interaction of *E. coli* Ssb protein with DNA occurs in the region between Trp-54 and Phe-60 (-Trp-54-His-55-Arg-56-Val-57-Val-58-Leu-59-Phe-60-). The space formed between these two amino acids could accommodate stacked nucleotide residues. This region is hydrophobic and α-helical with Trp-54 and Phe-60 separated by two helical turns [10]. The amino acids at binding site in mycobacterial Ssb are quite different to that observed in *E. coli*. Highly conserved His-55 is replaced by leucine in *M. tuberculosis* Ssb, similarly Trp 40, 54 and 88 are replaced by Ile 40, Phe 54 and 88 [124].

Ssb protein in other organisms forms different quaternary structures in eukaryotic cells, replication protein A (RPA) acts as a hetrotrimer [15]. Several bacteriophages and viral Ssb proteins function as monomer or dimer [119, 125]. Eubacterial Ssb proteins interact with many different other protein through its C-terminal acidic tail suggesting a conserved

mechanism by which other proteins can be recognized, recruited and bind Ssb. The C-terminal acidic tail generally ends in an Asp-Phe-Asp-Asp-Asp-Ile-Pro-Phe sequence in most studied *E. coli* Ssb protein and mainly contains Asp residues and is highly conserved among various life forms [126]. The C-terminal tail of Ssb is more prone to proteolysis and so is considered more dynamic in solution, moreover C-terminal tail is never visible in crystal structure further emphasizing it mobile nature [127-129]. Proteolysis of C-terminal tail has been reported on DNA binding and its deletion influences the stability of $(SSB)_{35}$ and $(SSB)_{65}$ binding modes [130]. Mutations at various positions in C-terminal leads to variety of defects in cells, ranging from temperature-sensitive lethality, hypersensitivity to DNA damage and reduced viability [131-134]. Deletion of C-terminal tail of Ssb is deleterious and renders the cell nonviable [135]. Phosphorylation of Ssb to regulate its function is common in eukaryotic and was also reported in prokaryotic Ssb. Tyrosine phosphorylation of *Bacillus subtilis* Ssb increased its *in vitro* affinity for ssDNA by 200 times [136].

1.7. Interaction of important proteins with Ssb.

Various proteins, which interact with Ssb proteins and mediate important aspects of DNA metabolism, are as follows:

1.7.1. *DNA polymerase III (χ-subunit).* DNA polymerase III holoenzyme binds to Ssb C-terminal tail through its χ-subunit. This interaction clears the potential Ssb proteins on lagging strand, facilitating replication of DNA [133]. This interaction also plays a crucial role in detaching primase from RNA primer, which can then be used by polymerase holoenzyme for replication [137]. Disruption in this binding is lethal to cells.

1.7.2. *Primase*. Primase is the only priming protein in bacteria [138], functioning in both leading and lagging strand during replication process [139]. Primase interacts with Ssb and strengthens the association between primase and RNA primer, its synthesis after which the protein is subsequently then disrupted further by DNA holoenzyme [137].

1.7.3. *RecQ DNA helicase*. RecQ helicase plays an important role in RecFOR pathway, Ssb is known to interact with winged helix subdomain of RecQ helicase by last 9 amino acids of its C-terminal tail. This interaction in turn stimulates the RecQ helicase activity required in recombination and repair processes [140].

1.7.4. *RecJ exonuclease*. RecJ exonuclease is a member of RecFOR pathway, it carries out nuclease activity in 5'-3' direction [141, 142]. Unlike other nucleases, Ssb interaction with RecJ nuclease increases its exonuclease activity [143]. RecJ plays an important role in genome maintenance as inactivation of RecJ nuclease leads to cell lethality [110].

1.7.5. *RecG helicase*. RecG has been known to stimulate multitude of genome maintenance activities including dsDNA recombination repair as well as ssDNA gap repair [107, 144], chromosome segregation [145], stabilization of stalled replication forks [146] etc. Ssb stabilizes RecG binding to negatively supercoiled DNA and stimulates its ATPase activity [147]. The interaction is mediated by the C-terminal region of *B. subtilis* Ssb [148].

1.7.6. *RecO protein*. RecO is the major mediator protein in RecFOR pathway. RecO binds to ssDNA and dsDNA and has DNA annealing activity. This annealing activity of RecO is

known to be stimulated by Ssb protein [149]. Ssb binds to RecO by it last eight amino acids and limits the formation of RecOR complex on ssDNA [150].

Other reported proteins which interact with Ssb in course of recombination and repair are PriA DNA helicase, PriB, exonuclease I, Uracil DNA glycosylase, DNA polymerase II, DNA polymerase V etc. [5].

1.8. Modulation of DNA repair activity of some proteins, without direct interaction with Ssb.

Ssb also modulates the activity of RecA and topoisomerase I enzyme indirectly by changing the topology of the DNA on which these enzymes act. This modulation in RecA and topoisomerase I activity may or may not necessarily involve direct interaction with Ssb protein.

1.8.1. *RecA protein*. Major Recombination process in living cells is mediated by RecA molecule. Recombination plays a prime role during DNA repair process [2]. Ssb protein plays major role in RecA mediated strand exchange as a mediator and facilitator protein. In strand exchange, RecA molecule transfers one strand from linear dsDNA substrate onto the single stranded substrate leading to formation of nicked circular molecule product. Ssb protein plays a very important role in the extension of RecA molecule onto ssDNA. RecA preferentially nucleates ssDNA substrate (slow step) at 5'end and then rapid extension occurs in 5'-3' direction [151]. This extension of RecA filaments is blocked if there are secondary structures on ssDNA as RecA by itself cannot remove secondary structure of ssDNA, leading to formation of abbreviated filaments that do not uniformly coat DNA. Ssb protein readily

removes such secondary structures following which the extending RecA readily displaces Ssb molecules, thus forming continuous filament on ssDNA substrate [21, 22, 152]. Ssb also binds to the displaced single strand after the strand exchange, thus preventing reversal of strand exchange reaction [153]. Generally Ssb protein does not interact with RecA protein but in mycobacteria it was shown that carboxy-terminal domain of Ssb interacts with cognate RecA [12].

1.8.2. *Topoisomerase I*. DNA topoisomerases change the topology of DNA by breaking and resealing of phosphodiester backbone. These are basically of two types, topoisomerase I breaks single strand at a time and allows passage of other strand, thus changing the linking number by one. Topoisomerase II breaks both the strands, allowing passage of entire duplex and thus changes the linking number by two. Ssb protein maintains single stranded DNA at the site of replication which acts as sink for Topoisomerase I and increase its activity without direct protein interaction with Ssb [25, 154, 155].

1.9. The Ssb protein of *Deinococcus radiodurans*.

Whole genome sequencing of *Deinococcus radiodurans* (strain BAA-816) predicted the presence of a *ssb* gene (DR0100) which required translational frame shift or RNA editing for the synthesis of complete functional protein. Immediately upstream of DR0100 was located one more *ssb*-like ORF (DR0099). Following re-sequencing of the DNA segment in strain ATCC13939 it was found that there was sequencing error of omission of two guanine nucleotides in the original submitted sequence. The complete ORF has now been submitted to Genbank (NC_001263) [14] and includes both original DR0099 and DR0100 ORFs. Our lab had resequenced the corresponding region from strain *Deinococcus radiodurans* strain BAA-816 to confirm the information derived from type strain ATCC13939. The complete sequence

of *ssb* gene has been submitted to gene bank (Accession number: JN571419). Complete ORF comprises of 301 amino acids and codes for a protein of approximately 32.6 kDa molecular mass. Its sequence is closely related to *Thermus thermophilus* and *Thermus aquaticus* Ssb. In solution condition it exists primarily as a dimer. It was also predicted to contain two OB folds, one at N-terminal and the other at C-terminal [14]. The sequence comparison of original and re sequenced *ssb* ORF is depicted in Fig 1.7.

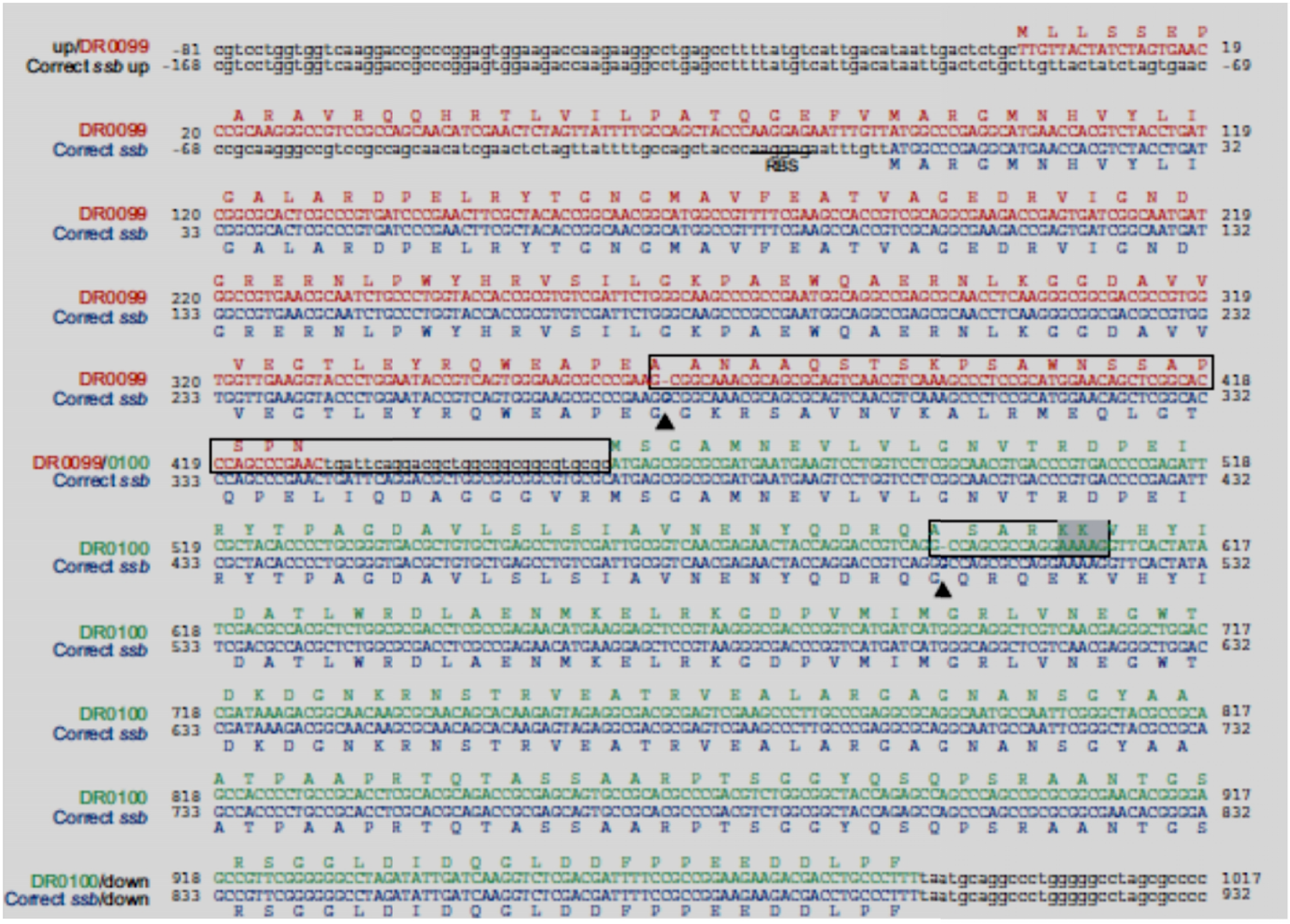


Fig. 1.7. Comparison of DNA sequence of original and resequenced *ssb* ORF. Original sequence is shown in red, while resequenced *ssb* ORF is shown in blue. Two omitted guainine nucleotides are shown by bold arrow. (Source : Eggington et. al, 2004 [14]).

The crystal structure of Deinococcal Ssb was obtained at 1.8 Å resolution by hanging drop vapor diffusion method [19]. It showed that Deinococcal Ssb comprises of two OB folds connected by β-hairpin motif. The asymmetry among two domains was predicted to contribute to some specialized role for each domain. The orientation of two OB folds was found to be different by 40° than that of *E. coli* counterparts. Dimeric interaction was mediated by extensive surface area of 1,288Å^2 contributed by N-terminal region and β-hair pin connector. It was also predicted that DNA binding was largely confined to C-terminal while protein-protein interaction between two Ssbs was mediated by N-terminal. The reported crystal structure of Deinococcal Ssb is shown in Fig 1.8

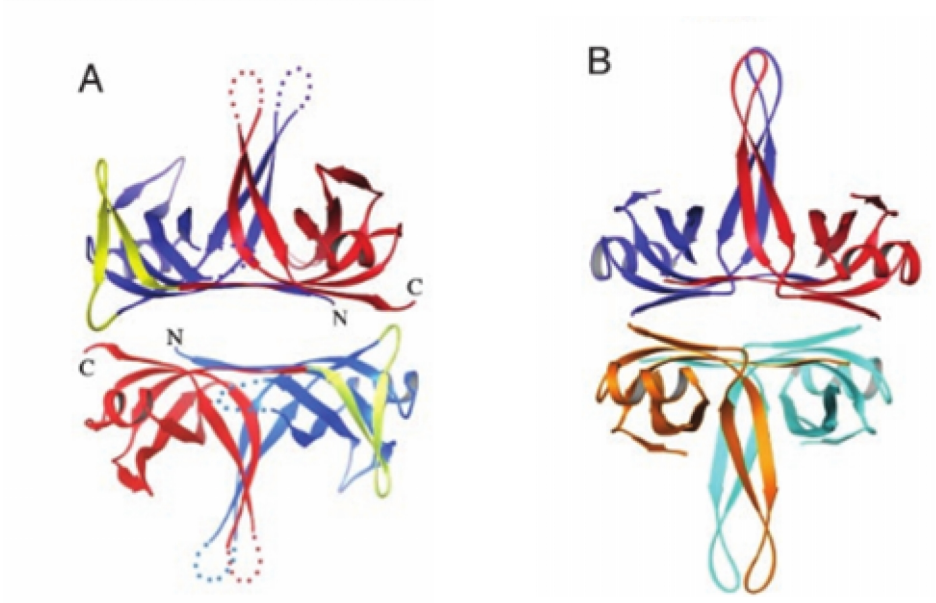


Fig. 1.8. Crystal structure of Deinococcal and *E. coli* Ssb. (A) Ribbon diagram of Deinococcal Ssb dimer, N-terminal region is shown by blue colour; C-terminal region is shown by red colour while connector region is shown by yellow colour. (B) Ribbon diagram of E. coli Ssb tetramer. Each protomer is colored differently. (Source: Bernstein et. al, 2004 [19]).

Crystal structure of Ssb protein complexed with homopolymeric dT_{35} ssDNA was obtained at a ratio of 1:2 and solved at 2.4Å resolution by molecular replacement method. The data showed that the binding mechanism of Deinococcal Ssb to ssDNA is similar to that seen in homotetrameric *E. coli* Ssb. One notable difference was the ordering of L_{45} loops of N-terminal, when bound to DNA suggesting that it plays some role in DNA binding [7]. The reported crystal structure of Deinococcal Ssb complexed with DNA is shown in Fig 1.9

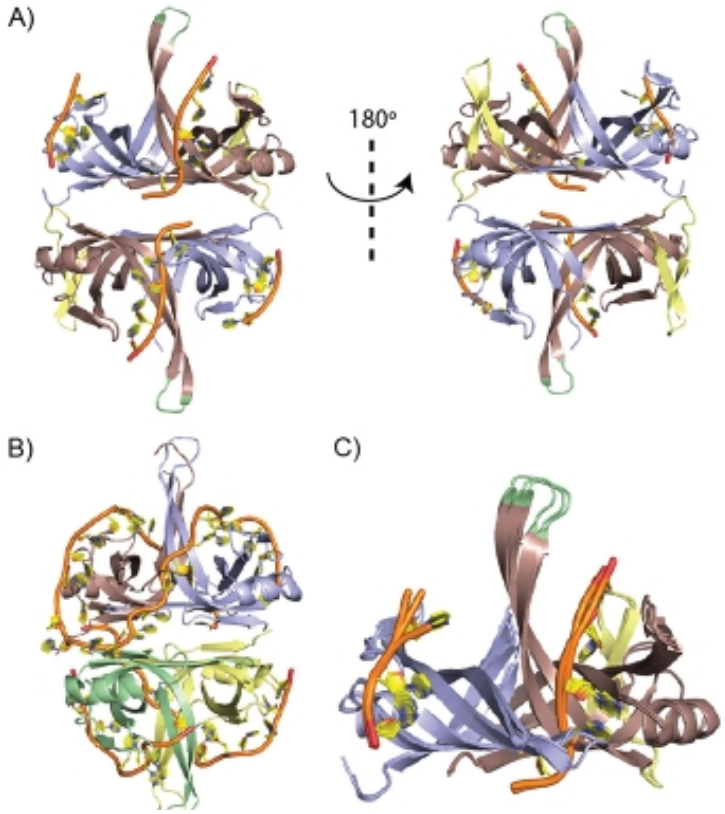


Fig. 1.9. Crystal structure of Deinococcal and *E. coli* Ssb complexed with DNA. (A) Ribbon diagram of DrSsb-ssDNA complex, DNA backbone (orange), N-terminal OB fold (salamon), C-terminal OB fold (light blue), beta hairpin linker (cream) and tip of L45 loop (green) is depicted. (B) Ribbon diagram of *E. coli* Ssb-ssDNA complex. (C) Overlayed DrSsb monomers bound to ssDNA, colored same as in (A) (Source: George et. al, 2012 [7]).

Biochemical studies have shown that the occluded binding site size for Deinococcal Ssb is 50±2 nucleotide on ssDNA and has a limited capacity to displace shorter strand in a duplex molecule. This would permit binding to ssDNA extension shorter than 26-30 nucleotides, which was not seen in *E. coli* [18]. The shift in binding mode upon changes in ionic condition is quite small in Deinococcal Ssb in comparison to its *E. coli* counterpart [17].

Ssb protein along with other repair proteins was also found in significant amount in the nucleiod of irradiated Deinococcal species [156]. *D. radiodurans* also expresses alternative Ssb like protein, DdrB. DdrB shows high induction following irradiation, has a preference for ssDNA and is a pentamer in solution [157], it also interacts with DrSsb protein and stimulates single strand annealing in its presence [158]. Following radiation stress DdrB is recruited early in the form of discrete foci in *D. radiodurans* nucleiod [156]. However, the lethality of Ssb deletion cannot be complemented by alternate Ssb like protein, DdrB in *Deinococcus radiodurans* indicating that the novel Ssb is indispensible [16].

Similar alternate Ssbs were also observed in *Bacillus subtilis* and *Streptomyces coelicolor*. In *B. subtilis* the alternate Ssb is induced from its own promoter in stationary phase in minimal medium [159] whereas in *S. coelicolor* alternate Ssb plays a role in chromosomal segregation during sporulation [160]. When Ssb levels are decreased in *Deinococcus radiodurans*, the resistance to irradiation and UV is also diminished [16]. Analytical ultracentrifugation studies have shown that Ssb protein forms globular dimers with some protrusions and in complementation assay Deinococcal Ssb can take-over the function of *E. coli* Ssb [161].

1.10. Effect of irradiation on gene expression in *Deinococcus radiodurans*.

The gene expression pattern following irradiation treatment was studied in *Deinococcus* species in detail by transcriptome and proteome analysis. Transcriptome analysis of exponentially growing cells irradiated to 3 kGy, induced 72 genes [162] while stationary phase cells irradiated to 15 kGy, up regulated 832 genes [47]. Some of the highly induced genes encode DNA repair proteins (*ddr*A, *ddr*B, *rec*A, *ssb*, *uvr*A, *ppr*A, *uvr*B, *gyr*A, *gyr*B etc.) and oxidative stress alleviating proteins (*kat*, *ter*B, *ter*Z, *msr*A, *dps*2) [47, 162]. Many genes which are induced following radiation stress do not necessarily contribute to radiation resistance, for example DRB0100, a homologue of eukaryotic DNA ligase B is highly induced following radiation but its mutant is not radiosensitive [32]. The proteome analysis done by 2D electrophoresis of Deinococcal proteins after irradiation displayed enhanced or *de novo* synthesis of at least 37 proteins in the first hour of recovery itself. The proteins, which were highly induced, belong to the category of DNA repair, oxidative stress and protein translation or folding. Ssb protein was shown to be highly induced and processed, followed by DdrB, The enhanced Ssb expression highlighted the importance of protecting single stranded DNA as the first line of defense in radiation damaged *D. radiodurans* [163].

1.11. Stress responsive transcriptional regulation in *Deinococcus radiodurans*

The efficient repair system found in *Deinococcus* group of bacteria comprises of efficient repair proteins to carry out error free repair of biomolecules as well as quick and prompt DNA damage response to upregulate the activation of repair proteins. Deinococcal DNA damage response comprises of several stress responsive *cis* elements and several transcriptional regulators.

1.11.1. *Radiation/Desiccation response motif (RDRM)*. The RDRM motif comprises of a 17 bp common palindromic sequence found upstream of set of radiation-induced genes found to be associated with DNA damage resistance. The conserved sequence of RDRM motif is displayed in Fig. 1.10

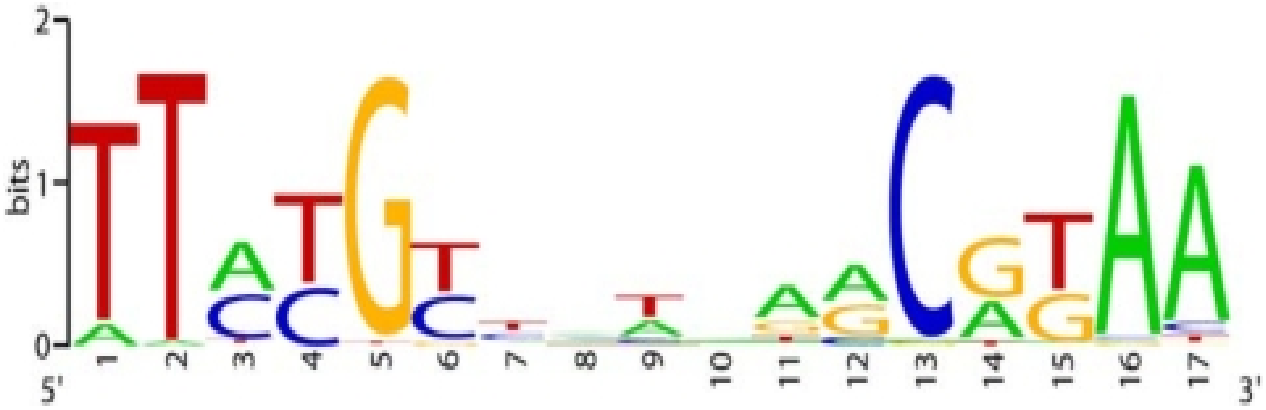


Fig. 1.10. Sequence of predicted RDRM. The height of the letter corresponds to the conserved position of the nucleotide across RDRM sequences found in 3 different Deinococcal species. (Source: Makarova et. al., 2007 [60]).

The centre of Dyad symmetry is found around the ninth nucleotide, which is T/A. The presence of RDRM has been reported in 29 genes of *Deinococcus radiodurans*, 25 genes of *Deinococcus geothermailis* [60] and 25 genes of *Deinococcus deserti* by de Groot et. al, 2009 [92] who preferred to call it radiation response motif (RRM). The RDR regulon, which is common to Deinococcal species, comprises of two groups: (1) a set of orthologous genes present in *Deinococcus radiodurans* and *Deinococcus geothermalis* that contain RDRM, and (2) a set of unique genes present in *Deinococcus radiodurans* that contains RDRM and are up regulated following radiation stress. The RDR regulon was predicted to comprise of 29 genes/operons mostly of DNA repair, recombination and replication in *Deinococcus*

radiodurans (*rec*A, *gyr*B, *ssb*, *sbc*D, *ruv*B, *ddr*A, *ddr*B, *ppr*A, *mut*S, *uvr*B, *uvr*C, *uvr*D, *mut*L, *gyr*A, *rec*Q etc.) [60]. The RDRM and RDRM like sequences based regulation of *ssb* gene expression was recently demonstrated experimentally for various DNA damaging stresses [164].

1.11.2. *Transcriptional regulators*. The well known stress-responsive transcriptional regulators encoded by Deinococcal genome are DdrO, IrrE, DrRRA and OxyR. DdrO is regarded as a global regulator of the RDR regulon present in *Deinococcus radiodurans*, as it is the only gene for the predicted transcriptional regulator that is preceded by RDRM (radiation desiccation response motif) site [60]. IrrE (also called as PprI) is regarded as a general switch that activates a series of repair pathways for efficient repair of DNA following radiation stress [165]. It upregulates the expression of 31 proteins comprising of common DNA repair proteins (RecA, PprA and Ssb), transcription, translation, protease, chapereones, signal transduction, cell cycle control and down regulates expression of 4 proteins [165]. DrRRA regulates the expression of many genes related to DNA damage (*ppr*A, *cin*A, *rec*A, *lig*T, *gyr*B and *uvr*B), antioxidant proteins, proteases, chaperones etc. [79]. OxyR regulates the activity of catalase and ROS scavenging activity under hydrogen peroxide stress [80, 166].

1.12 Present work

Present work was initiated to understand (a) the significance of N & C-terminal OB fold separately and in combination versus the full length Ssb, and its modulatory effect on activity of other repair proteins (b) The modulation in Ssb expression, post-stress condition. The work was carried out with following defined objectives:

1. Cloning and over expression of full length *ssb* gene (Ssb_{FL}) as well as truncated Ssb genes, encoding N terminal (with or without connector sequence [Ssb_{NC} & Ssb_{N}]) and C terminal halves (Ssb_{C}) in *E. coli* BL-21 strain and purification of corresponding proteins.

2. Cloning, over-expression and purification of Deinococcal RecA for conducting strand exchange assays with the purified Ssb protein.

3. Biochemical characterization of N (with or without connector sequence) and C terminal halves of Ssb protein, *vis-a-vis* the full length Ssb protein in terms of DNA binding, topoisomerase assays and trimerization or strand exchange assays with cognate RecA, and *E. coli* RecA.

4. Regulation of Deinococcal Ssb protein expression in response to DNA damaging stresses and importance of Radiation Desiccation Response Motif (RDRM) elements present in the promoter region in regulation.

The thesis is organised into following 4 chapters:

Chapter 1: General introduction

This chapter describes general information about repair pathways adopted by *Deinococcus radiodurans* in combating various DNA damaging stresses and summarizes what is known about radiation-responsive gene expression in this microbe.

Chapter 2: Materials and Methods

This chapter describe the materials, their sources and various experimental techniques used in this study.

Chapter 3: Results and Discussion

This chapter describes the results obtained from the study and discusses their probable interpretation for the overall function of Deinococcal Ssb protein. The chapter has been subdivided into 3 sections.

3.1. Cloning, over-expression and purification of Deinococcal full length protein (Ssb_{FL}), N-terminal OB domain (Ssb_N), N-terminal OB domain with connector (Ssb_{NC}), C-terminal OB domain (Ssb_C) and RecA protein and characterization of various Ssb variants for their oligomeric status.

3.2. Analysis of functional roles of various Ssb variants or their combinations in DNA binding, physical and functional interaction among Ssb variants, promotion of topoisomerase activity and trimerization assays with cognate Deinococcal RecA or non cognate *E. coli* RecA.

3.3 Effect of various DNA damaging stresses on *in vivo* Ssb protein expression and elucidation of role played by RDRM sequences in radiation induced gene expression.

Chapter 4: Summary and Conclusion

This chapter summarises the important conclusions of the study. It attempts to assign role to each Ssb domain in carrying out various biochemical activities and highlights the role of RDRM in Deinococcal gene expression.

Chapter 2
Materials and Methods

2.1. Growth media and culture conditions.

Deinococcus radiodurans used in this study was grown on TGY medium (1% Tryptone, 0.5% Yeast extract, 0.1% Glucose) at 32°C, maintained under agitation at 150 rpm or on TGY agar plates. *E. coli* (strains DH5α, ER2566, STL2669 and BL-21pLys) used in this study were grown on Luria broth (LB) medium (1% Tryptone, 0.5% Yeast extract, 1% NaCl) at 37°C, maintained under agitation at 150 rpm or on LB agar plates. Cultures of recombinant *E. coli* strains carrying different plasmids (Table 2.1) were supplemented with carbenicillin at 100 µg/ml final concentration. Bacterial stock cultures were maintained in two ways. The working culture was plated onto the agar plate containing TGY (*D. radiodurans*), grown for 48 h at 32°C or on LB agar plates (*E. coli*) and grown at 37°C for 12 h. The working stock was stored at 4°C for not more than 7 days. The long term storage of all bacterial strains were carried out by dispensing 10^9 cells of each strain in cryotubes with 20% glycerol as cryoprotectant, snap frozen in liquid nitrogen and stored at -70°C.

2.2. Genomic DNA isolation from *Deinococcus radiodurans*.

The genomic DNA was prepared from *Deinococcus radiodurans* as described earlier [167] with some modifications. Overnight grown culture of *Deinococcus radiodurans* (20ml) were centrifuged at 13000 rpm for 5 mins at 4°C and first washed with 0.9% NaCl. Following centrifugation, the pellet formed was further washed twice with absolute ethanol to remove outer membrane and pigments. The resulting pellet of ethanol-stripped cells was allowed to stand at 37°C for 5mins to evaporate any residual ethanol. The cell pellet was subsequently resuspended in 0.1ml of TE buffer (10mM Tris-HCl and 1mM EDTA pH 8.0). The cells were then disrupted by lysozyme treatment (final concentration 10 mg/ml) at 37°C for 1h.

The proteins present in cells were removed by proteinase K solution (2% sodium dodecyl sulphate, 0.1M EDTA, pH8.0, 4 mg of proteinase K) treatment at 50°C for 3h. A solution of Phenol: chloroform at a ratio of 1:1 was then added to the mixture and aqueous phase was extracted twice in a fresh tube. DNA was precipitated by adding 0.1 ml of 3M sodium acetate (pH 7) and 2 ml of ice-cold absolute ethanol. The DNA was spooled out with a curved glass rod. The spooled DNA was then washed twice with 70% ethanol, air dried in vacuum and resuspended in 1000 µl of TE buffer and stored at 1µg/ µl final concentration. The purity of DNA was quantitated by taking absorbance at 260 and 280nm. The ratio obtained was 1.78 indicating the purity of sample. Alternatively, the genomic DNA was also prepared using Genomic DNA preparation kit (Himedia, India) as per manufacturer's protocol.

2.3. PCR amplification.

The primers required for amplification of different genes were designed based on the nucleotide sequence of concerned genes available in NCBI database (www.ncbi.nlm.nih.gov/nucleotide/). The primers were designed based on the sequences for forward and reverse strand. GC percentage, T_M values, secondary structure and dimer formation among primers were analysed and calculated using DNA MAN software (Lynnon Biosoft, Germany). Sequences for appropriate restriction endonuclease site were incorporated at 5' end of each primer.

PCR amplification was carried out using genomic DNA isolated from *Deinococcus radiodurans*. Primers were added at 0.2 µM concentrations, while dNTPs were maintained at 200 µM concentrations. As Deinococcal genome is GC rich, the amplification

was done by using combination of Taq DNA polymerase and Tgo polymerase in presence of GC rich resolution buffer (GC rich amplification kit, Roche). The amplification was carried out for 30 cycles involving denaturation at 95°C, annealing in the range of 52-61°C depending on individual primer and extension at 72°C. The products obtained were analysed by agarose gel electrophoresis. The PCR products obtained were resolved on 0.8% agarose gels in Tris-borate-EDTA buffer (TBE) (0.09 M Tris, 0.088 M boric acid and 0.002 M EDTA, pH 8.0) containing 0.5 µg/ml concentration of ethidium bromide and visualized under UV illumination. The amplicon was excised from the gel. The DNA was isolated from the gel piece using affinity columns (Gel extraction kit, Qiagen, Germany) by following the protocol as described by the manufacturer.

2.4. Restriction digestion and electrophoresis of DNA.

Restriction endonuclease digestion was carried out as per the manufacturer's protocol (New England Biolabs Ltd., UK). The digested DNA was resolved on 0.8% agarose gels containing 0.5 µg/ml ethidium bromide, prepared in 1X TBE buffer. Electrophoresis was carried out at 8 V/cm and DNA fragments were visualized under UV on a transilluminator. The DNA markers (100bp and 1kb ladder) used in this study were obtained from NEB.

2.5. Ligation of restriction digested DNA fragments to respective vectors.

The ligation reaction was carried out by using quick DNA ligase (T4 DNA ligase, Roche biochemicals, India) as per the manufacturer's protocol. The insert to vector ratio was maintained at 3:1 for sticky end ligation or at 1:1 for blunt end ligation. Total DNA concentration was kept at 200 ng/20 µl. The reaction was carried out in ligase reaction

buffer, in presence of ATP by overnight incubation at 20°C as per manufacturer's protocol. After incubation period, the ligation mix was directly used for transformation.

2.6. Plasmid DNA isolation.

The various plasmids used in this study are listed in Table 2.1. Plasmid DNA from various *E. coli* strains were isolated by using either alkali lysis method [168], or by using QIA prep Spin Miniprep Kit (Qiagen, Germany) as per the manufacturer's protocol. Briefly, the cells were first resuspended in isotonic solution and then disrupted using strong alkaline solution of SDS and NaOH and incubated on ice for few minutes. Next potassium acetate solution is added to separate plasmid from chromosomal DNA. Followed by centrifugation step the supernant is transferred to miniprep column (Qiagen, Germany), centrifuged at 13K for 1 min for plasmid to bind to the column. The column is subsequently then washed twice to remove as much salt possible and then eluted by adding TE buffer.

2.7. Transformation of *E. coli* cells

Competent *E. coli* cells of various strains were made by calcium chloride-rubidium chloride method. Overnight grown cells were diluted 100 fold in fresh LB and allowed to recover at 37°C with agitation at 150 rpm up to 0.3-0.5 OD_{600nm}. Cells were centrifuged in cold and washed with solution A (10 mM MOPS, pH 7.0, 10 mM RuCl) and further incubated with solution B (100 mM MOPS, pH 6.5, 50 mM $CaCl_2$ and 10 mM RuCl) for 1 h at 4°C. After incubation, the cells were centrifuged and resuspended in fresh solution B containing 15-20% glycerol and stored in small aliquots of 150-200 µl for immediate use or snap frozen in liquid N_2 and stored at -70°C. Transformation of *E. coli* cells was carried out by heat shock

method. Briefly 100 ng of the required plasmid DNA was incubated with 150-200 µl of competent cells for 1 h at 4°C. The heat shock was given for 1.5 min at 42°C, followed by 2 min incubation on ice. The recovery of cells was done by addition of 1 ml LB media and further incubating it at 37°C for 1 h. The cells were subsequently gently pelleted by centrifugation at 5000 rpm for 3 mins. The pellet was redissolved in fresh 100 µl of LB and plated on LB agar plates containing appropriate antibiotics. The plates were incubated overnight at 37°C and transformants so obtained were scored and characterized.

Table 2.1: List of strains, plasmids and primers used in this study

Host cells, Plasmid or primer	Description	Reference, source or remarks
Bacterial Strains *Deinococcus radiodurans*	*Deinococcus radiodurans* R1, ATCC BAA-816	Lab collection
E. coli DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	Lab collection
E. coli BL-21pLysS	F^- ompT gal dcm lon $hsdS_B(r_B^-$ $m_B^-)$ λ(DE3) pLysS(cm^R)	Novagen
E. coli ER2566	*fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10*--Tet_s*)2 [dcm] R(zgb-210::Tn10*--Tet_s) *endA1* Δ*(mcrC-mrr)114::IS10.*	NEB
E. coli STL2669	(Δ(*recA-srlR*)306:*Tn*10 *xonA*2(*sbcB*$^-$))	Lab collection
E. coli $BLSsb_{FL}$	*E. coli* BL-21pLysS cells containing recombinant plasmid pET16b- Ssb_{FL}	This study
E. coli $BLSsb_C$	*E. coli* BL-21pLysS cells containing recombinant plasmid pET16b- Ssb_C	This study
E. coli $ERSsb_{NC}$	*E. coli* ER2566 cells containing recombinant plasmid pTWIN1- Ssb_{NC}	This study
E. coli $ERSsb_N$	*E. coli* ER2566 cells containing recombinant plasmid	This study

	pMXB10- Ssb_N	
E. coli STLRecA	*E. coli* STL2669 cells containing recombinant plasmid pET16b-RecA devoid of any His-Tag	This study
Plasmids		
pET16b	Amp^r, protein expression vector having His-Tag	Novagen
pTWIN1	Amp^r, Protein expression vector having intein Tag.	NEB
pMXB10	Amp^r, Protein expression vector having intein Tag	NEB
$pETSsb_{FL}$	Deinococcal Ssb_{FL} gene cloned at *Nde*/*Bam*HI restriction sites.	This study
$pETSsb_C$	C-terminal region of deinococcal Ssb cloned at *Nde*/*Bam*HI	This study
pETRecA	Deinococcal RecA gene cloned at *Nco*I/*Bam*HI restriction sites.	This study
$pTWINSsb_{NC}$	N-terminal with connector region of deinococcal Ssb cloned at *EcoR*I/*Bam*HI restriction sites.	This study
$pMXB10Ssb_N$	N-terminal region of deinococcal Ssb cloned at *Nde*I/ *Xho*I restriction sites.	This study
Primers		
FLF	5-CCAGGGATCCAAGGAGAATTTGTTCATATGG-3	*Bam*HI, *Nde*I
FLR	5-CGCTGTTTCCTTGCTGGATCCTGTTG-3	*Bam*HI
CTF	5-TGATTCAGGATCCTGGCGGCGGCGTGCATATGAGC-3	*Nde*I
CTD	5'GCTGGGTCATGTTGGGGATCCTTGGTG-3'	*Bam*HI
NCF	5-GGAATTCCATATGGCCCGAGGCATGAAC-3	*Eco*RI
NCD	5-CGGGATCCTTAGCGCACGCCGCCGCC-3	*Bam*HI
NF	5-GGAATTCCATATGGCCCGAGGCATGAAC-3	*Nde*I
ND	5-CCGCTCGAGTTCGGGCTGGGTGCC-3	*Xho*I
RF	5-CATGCCATGGCCATGAGCAAGGACGCC-3	*Nco*I

RD	5-CCGCTCGAGGTTTACGCTTCGGCGGC-3 Restriction site contained in primer is underlined.	*Xho*I

2.8. Labeling of DNA by non-radioactive DIG label.

Labeling of dT50 oligo was carried out by using Digoxigenin labeled ddUTP and terminal transferase enzyme (Roche, India) as per the manufacturer's protocol. Briefly, 100 pmol of dT50 oligo was incubated in reaction buffer (0.2 M potassium cacodylate, 25 mM Tris-HCl, 0.25 mg/ml BSA pH 6.6, provided by manufacturer) with 5 mM $CoCl_2$, 0.05 mM of DIG labeled ddUTP and 20 units of terminal transferase for 1 hour at 37°C. Reaction was terminated by adding EDTA (0.02 M, final concentration). The labeled oligos were purified by passing through mini superdex 50 columns (Roche, UK) and used in electrophoretic mobility shift assay.

2.9. Electro blotting of DNA on nylon membrane.

DNA-protein complexes were electro blotted on to nylon membrane in the presence of 0.5X TBE buffer. The gels were carefully sandwiched with nylon membrane and care was taken to avoid any air gap in between. The transfer was carried out for 30 mins at 400 milliamps.

2.10. DNA manipulations for protein overexpression and purification.

2.10.1. Cloning of *ssb* variants (ssb_{FL}, ssb_C, ssb_{NC} and ssb_N) and *recA* genes

The oligonucleotide primers and plasmids used and recombinant strains generated are described in Table 2.1. PCR amplifications were carried out from *D. radiodurans* (R1)

genomic DNA. The full length *ssb* gene (Ssb_{FL}) was amplified using specific primers FLF and FLR. The 1 kb product was digested with *Bam*HI and ligated to pUC19 at *Bam*HI site to obtain the plasmid pUC19Ssb_{FL}. The insert from pUC19Ssb_{FL} was released by digesting it with *Nde*I and *Bam*HI and ligated to pET16b at identical sites to generate the plasmid pETSsb_{FL} for Ssb_{FL} overexpression. Ssb-C-terminal domain (Ssb_C) was PCR amplified by using primers CTF and CTD. The 0.64 kb product was restriction digested with *Nde*/*Bam*HI and cloned at identical sites in pET16b to obtain the plasmid pETSsb_C for Ssb_C overexpression. Ssb-N-terminal with connector was amplified by using primers NCF and NCD. The 0.372 kb product was restriction digested with *Eco*RI and *Bam*HI and cloned in pTwin1 vector at identical sites to generate the plasmid pTwinSsb_{NC} for Ssb_{NC} overexpression. Ssb-N-terminal region without connector was amplified using primers NF and ND. The 0.342 kb product was restriction digested with *Nde*I and *Xho*I and cloned in pMXB10 vector at identical sites to construct plasmid pMXB10Ssb_N for Ssb_N overexpression. *D. radiodurans recA* gene was amplified by using primers RF and RD. The 1.1 kb product was restriction digested with *Nco*I/*Xho*I and cloned in pET16b at *Nco*I (to avoid His tag) and *Xho*I sites to generate plasmid pETRecA for RecA overexpression. The identity and sequence accuracy of all the clones was ascertained by suitable restriction digestion and DNA sequencing of insert.

2.10.2. Overexpression and purification of recombinant Ssb_{FL} and Ssb_C proteins.

The constructs pETSsb_{FL} and pETSsb_C were transformed separately into *E. coli* BL-21(pLysS) cells to obtain strains BLSsb_{FL} and BLSsb_C. Cells were grown in LB medium at 37°C and induced by the addition of Isopropyl β-D-1-thiogalactopyranoside (IPTG) to a

final concentration of 1 mM for 4h. The His-tagged proteins were purified from cell free extracts using Ni-NTA (nickel-nitrilotriacetic acid) resin and eluted with an imidazole gradient (1 → 500 mM). Individual proteins were dialyzed overnight to remove imidazole. Trace amount of protein contaminants were removed by passing the proteins through the Q-sepharose affinity matrix and eluting with NaCl gradient (20 mM→1000mM). The homogeneous fractions which were devoid of both endo- and exonucleases were pooled and dialyzed in the Ssb storage buffer (20 mM Tris-HCl pH 8.3, 500 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol and 50% glycerol), snap-frozen in liquid nitrogen and further stored at -70°C.

2.10.3. Overexpression and purification of recombinant Ssb_{NC} and Ssb_N proteins.

The pTwinSsb_{NC} and pMXB10Ssb_N constructs were separately transformed into *E. coli* strain ER2566 to obtain ERSsb_{NC} and ERSsb_N strains. Cells were grown in LB medium at 37°C and induced by adding IPTG to a final concentration of 1mM at 20°C for 12 h. The clarified cell free extracts (in 20 mM Tris-HCl pH 8.5, 0.5 M NaCl) were loaded onto a chitin column and washed with 20 volumes of column buffer (20 mM Tris pH 8.5, 0.5 M NaCl). On-column cleavage of Ssb_{NC} was performed by equilibrating the column with 20 mM sodium phosphate buffer pH 6.0 containing 0.5 M NaCl and incubating it at 4°C for 4 days. For Ssb_N, column was equilibrated with 20 mM Tris-HCl, 0.5 M NaCl pH 8.5 buffer containing 10 mM DTT (dithiothreitol) and incubated overnight at 4°C. Bound proteins were eluted from column by passing equilibration buffer without DTT. The eluted fractions were analyzed by 14% SDS-PAGE. To remove trace amount of impurities, the eluted fractions of Ssb_{NC} or Ssb_N were passed through a Q-Sepharose column and eluted by a NaCl

(20 mM →1000 mM) gradient. The fractions free of both endo- and exonucleases were pooled and dialyzed in Ssb storage buffer, snap-frozen in liquid nitrogen and stored at -70°C.

2.10.4. Over expression and purification of recombinant RecA protein.

Deinococcal RecA protein was purified as described previously [169] with some modifications. Briefly, pETRecA construct was transformed into *E. coli* STL2669 cells to obtain strain STLRecA. RecA was over expressed by adding IPTG (1 mM final concentration) at 37°C for 4 h. Cells were lysed by sonication and the crude lysate was centrifuged at 40,000g for 1 h. RecA protein was precipitated by adding 10% Polymin-P (pH 7.9) to a final concentration of 0.5% over 15 min with continuous stirring. The pellet was extracted until most of polymin-P was removed, as described [169]. Other trace contaminating proteins were removed by Q-Sepharose column chromatography and eluted with a NaCl gradient (20 mM → 1000 mM). The nuclease-free protein aliquots were dialyzed overnight in RecA storage buffer (20 mM Tris-HCl pH 7.5, 1 mM DTT and 10% glycerol) and stored at -70°C.

2.11. Determination of oligomeric status of purified proteins by gel exclusion chromatography and glutaraldehyde cross-linking.

Oligomeric status of all Ssb variants was determined by Superdex-75 gel exclusion chromatography having a matrix of cross-linked agarose and dextran with a bed volume of 24 ml (GE Health care, UK). Standard graph for the column was prepared based on the elution volume obtained for Bovine serum albumin (66kDa), Ovalbumin (44 kDa),

Carbonic anhydrase (29 kDa) and Cytochrome-C (12.4 kDa) protein standards. Approximately 250 µl of each Ssb variant (~ 2.5 mg/ml) was individually passed through the column. The flow rate was maintained at 0.5 ml per min. Ssb_{FL} or Ssb_{C} eluted as single major peak (fraction F1). Ssb_{N} or Ssb_{NC} formed two major peaks. First peak (high molecular mass) eluted in the void volume of the column (fractions F1 and F2), while the second peak was collected as fraction F3. All fractions obtained, were resolved by 14% SDS-PAGE.

Determination of the oligomeric status of Ssb was also carried out by glutaraldehyde cross linking. Briefly, 25 µg of individual protein was mixed with 5 µl of freshly diluted 2.5% glutaraldehyde in 50 µl reaction mixture containing 50 mM sodium phosphate buffer, pH 8.0 and incubated at 37°C for 5 min. The reaction was terminated by adding 10 µl of 1M Tris-HCl pH 8.5, and then resolved by 14% SDS PAGE and stained with Coomassie Brilliant Blue (CBB-G-250) dye.

2.12. Protein estimation.

The protein estimation was carried out as described earlier [170]. Briefly the purified protein sample or cellular crude extract was added to 500 µl of deionized water. The protein was subsequently precipitated by adding 50 µl of 1.5 mg/ml of DOC (Deoxycholate, Sigma, India) and 50 µl of 92% TCA solution (Trichloro acetic acid, SRL, India), followed by centrifugation at 13000 rpm for 15 mins. The precipitated protein was subsequently again resuspended in 500 µl of deionized water. Then 500 µl of Lowry's reagent (Sigma Aldrich, India) was added to the sample and allowed to incubate for 15 min. After incubation, 250 µl of Folin-C solution (18 ml of 2N Folin-C and 90 ml of water) was added to the reaction mix

and allowed to incubate for 30 min. The samples were analyzed spectrophotometrically at 750 nm. The linear standard graph of protein concentration was made by using defined concentration of BSA as control.

2.13. SDS-PAGE analysis of proteins.

The reagents used for casting of SDS-PAGE gels, electrophoresis and staining of proteins in gel are listed in table 2.2

Table 2.2: List of composition of common solutions used in SDS-PAGE.

Reagent	**Composition**
30% Acrylamide stock solution	29.2% Acrylamide, 0.8% Bis-acrylamide dissolved in 100 ml distilled water and filtered.
1.5 M Tris, pH 8.8	18.3g Tris in 100ml deionized water and pH maintained by HCl
0.5 M Tris, pH 6.8	6.1g Tris in 100ml deionized water and pH maintained by HCl
10% SDS (Sodium dodecyl sulphate)	10g SDS in 100ml of deionized water
10% APS (Ammonium per sulphate)	1g APS in 10ml of deionized water
TEMED (Tetramethylethylenediamine)	absolute
Running buffer	25 mM Tris, 192 mM glycine, 0.1% SDS

Destaining solution (DS)	10% Methanol, 10% acetic acid and 2% glycerol
Coomassie Brilliant Blue G250 (CBB)	0.2% Coomassie Brilliant Blue G250 in 40% Methanol and 20% Acetic acid
2X Cracking buffer (10ml)	460mg SDS, 7.6mg EGTA, 20mg sodium azide, 2ml glycerol, 200 µl of 0.1% bromophenol blue, 2.5 ml of 0.5 M Tris pH 6.8, 1 ml of beta mercaptoethanol and 2 mg of PMSF

SDS- PAGE analysis is an important technique for resolving proteins under denaturing conditions. The gel solution was made by appropriately diluting stock solution of 30% acrylamide (29.2% Acrylamide, 0.8% Bis-acrylamide), 1.5 M Tris-HCl pH 8.8 and 10% SDS to get desired percentage of resolving gels (12-14%), 375 mM Tris-HCl pH 8.8 and 0.1% SDS. APS (0.1% final concentration) and TEMED (10 µl per 20 ml of gel mix) were used as free radical generator and catalyst respectively. The gel solution was poured between two glass plates (10X8 cms) having 1 mm thickness and allowed to polymerize for at least 1 hr. The top portion of gel was buffered with water saturated butanol or isopropanol to avoid contact with air. After resolving gel polymerization, the top layer of water saturated butanol was washed off by distil water. The stacking gel solution (4% acrylamide/bisacrylamide, 125 mM Tris-HCl pH 6.5 and 0.1% SDS, final concentration) was casted on top of resolving gel by appropriately diluting stock solution of 30% acrylamide, 0.5 M Tris-HCl pH 6.5, 10% SDS and allowed to polymerize for at least 0.5 h. Appropriate wells were generated while pouring stacking gel solution by using standard sized combs. After incubation of 0.5 h the space between the gel caster and combs was

flushed with distilled water and then comb was removed slowly to avoid any damage to wells. The wells thus formed were rinsed twice with distill water to flush out any loose gel pieces. The gels which had proper wells were subsequently used for resolving protein under denaturating conditions. The proteins loaded per lane were electrophoretically resolved at 100V till dye front reached stacking gel boundary and subsequently at 200V till dye front reached resolving gel boundary. After electrophoresis the gels were stained with Coomassie Brilliant Blue (CBB-G-250) dye and destained with destaining solution (DS). Protein marker SDS-7 (Sigma chemical company, India) was used for molecular mass estimation.

2.14. Electroblotting of proteins on nitrocellulose membrane.

Electroblotting of SDS-PAGE resolved proteins was carried out on nitrocellulose membrane. The blotting was carried out for 1.5 h at 400 milliamps in transfer buffer (25 mM Tris pH 8.3, glycine 150 mM). The gels were sandwiched between nitrocellulose membrane on positive side and blotting sheet on the negative side. Care was taken to avoid any air bubble. After blotting the membranes were reversibly stained with Ponceau-s stain (Sigma Aldrich co. India) to confirm uniform transfer of proteins. The stain was subsequently removed by rinsing with distil water three times.

2.15. Antibody generation against Deinococcal Ssb and RecA proteins.

Purified Ssb and RecA proteins were individually resolved by 12% SDS PAGE. One lane of the gel was stained with Coomassie Brilliant Blue G250 to locate the position of protein in other lanes. The gel at that position was excised with a sterile blade. The excised gel slice was cut in to small pieces and incubated with Tris-NaCl (20mM Tris pH 8.0, 50mM NaCl)

buffer for protein elution. The incubation was carried out overnight at 20°C under mild (50-60 rpm) agitation. The eluted proteins were checked for concentration and quality by Lowry's method of protein estimation followed by 12% SDS-PAGE respectively. The eluted proteins were lyophilized and 8-10 µg of both proteins (RecA and Ssb) were given to a commercial entity (Bangalore Genei Bangalore, India and Invenio Life Technologies, India) for antibody generation. The antibody generation required at least two booster doses of protein injection to rabbit. The polyclonal antibody thus generated, was checked for specificity and affinity by western blotting and immunodetection analysis, as described below.

2.16. Western blotting and Immunodetection of Deinococcal Ssb and RecA proteins.

The immunodetection of Ssb and RecA proteins from cellular extracts of *Deinococcus radiodurans* was carried by using specific primary polyclonal antibody generated against the individual proteins in rabbit. The crude cellular extracts of *Deinococcus radiodurans* were first resolved by 12% SDS-PAGE, followed by electroblotting on nitrocellulose membrane. The membrane was first incubated in blocking solution [1% of blocking reagent (Roche, UK) in 100 mM maleic acid, 150 mM NaCl buffer, pH 7.5] for 1 h. Then the membrane was incubated with primary antibody for 12 h in blocking solution. Subsequently, the membrane was washed three times in TS buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl.) followed by incubation with secondary antibody (anti rabbit IgG raised in goat and coupled to alkaline phosphatase) for 1.5 h. The membrane was washed twice and developed by using NBT /BCIP (nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate) as chromogenic substrate.

2.17. Native PAGE for DNA:protein complexes.

Native PAGE analysis is an important technique of resolving DNA-protein complexes under native conditions. The 12% gels were casted using acrylamide solution (29.2% Acrylamide, 0.8% Bis-acrylamide) and 40 mM Tris acetate pH 7.4. The gel solution was made by appropriately diluting stock solution of 30% acrylamide and 1 M Tris acetate pH 7.4. APS (0.1% final concentration) and TEMED (10 µl per 20 ml of gel mix) were used as free radical generator and catalyst respectively. The gel solution was then poured between two glass plates (10X8 cms) having 1 mm thickness. Appropriate wells were generated while pouring solution by using standard sized combs. The gel was allowed to polymerize overnight at room temperature. Next day, the space between the gel caster and combs was flushed with distilled water and then comb was removed slowly to avoid any damage to wells. The wells were rinsed twice with distill water and used for resolving DNA-protein complexes.

2.18. Electrophoretic Mobility Shift Assays (EMSA).

EMSA was carried out with individual Ssb_{FL}, Ssb_{C}, Ssb_{NC} or Ssb_{N} and DIG-labeled dT50 oligo. Individual protein was incubated with the dT50 oligo in 20 mM Tris-acetate pH 7.4 and 1 mM $MnCl_2$ at 27°C for 20 min. The DNA-protein complexes were resolved by 12% native PAGE for 5 h at 50 V in 40 mM Tris acetate buffer, pH 7.4. DNA-protein complexes were electroblotted onto nylon membrane, cross-linked by UV exposure, probed with anti-DIG antibody coupled with alkaline phosphatase and developed using NBT-BCIP (nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate) chromogenic substrate. The

bands visualized were quantitated by Gel Quant software (Biosystematica, UK) and data were fitted to Hill's equation (Origin Pro 8 analysis software, http://www.originlab.com/index.aspx?go=PRODUCTS/OriginPro) to obtain K_D values for Ssb_{FL} and Ssb_C proteins. Each experiment was repeated three times and K_D values were obtained from each experiment. The K_D values (with standard deviation) were calculated by taking average of three independent experiments. EMSA, as described above, was also carried out with different combinations of Deinococcal Ssb variants (Ssb_{NC}/Ssb_N and Ssb_C) and also with combination of Ssb_{NC}/Ssb_N and *E. coli* Ssb.

2.19. Domain interaction studies.

Purified Ssb_C was bound to Ni-NTA matrix through its His tag until saturation had been achieved by analyzing small portion of gel matrix by 14% SDS-PAGE. Ssb_{NC} or Ssb_N was then added to Ni-NTA attached Ssb_C individually. As a control, Ssb_{NC} or Ssb_N was also incubated separately with Ni-NTA matrix to check for non-specific binding. The columns were washed with increasing concentration of immidazole (0 → 200 mM) and final elution was done with 250 mM immidazole. The elutants so obtained were resolved by 14% SDS PAGE and stained with CBB. In reverse binding method, Ssb_N (without connector region) with chitin binding domain was bound to chitin beads and *E. coli* crude extract containing over expressed Ssb_C protein was added to and equilibrated in the column. *E. coli* crude extract containing over expressed Ssb_C was purposefully used in this experiment to provide more competition to N-terminal in binding C-terminal. The column was subsequently washed and tag of N-terminal was removed by overnight treatment with 10 mM DTT. The eluting fractions contained both N-terminal and C-terminal portions. These two experiments

provided additional proof that N-terminal portion physically interacts with C-terminal portion of Ssb.

2.20. Stimulation of *E. coli* topoisomerase I activity by Deinococcal Ssb variants.

The effect of various Deinococcal Ssb variants on *E. coli* topoisomerase I activity was investigated, as described earlier [24]. The reaction was carried out in a buffer provided by the supplier (NEB) with 0.5 µg of M13mp18 negatively superhelical (form I) DNA, 0.1 unit topoisomerase I and indicated concentration of individual Ssb variants or their combination at 37 °C for 15 min. The reaction was terminated by the addition of EDTA to a final concentration of 10 mM and reaction further incubated at 65°C for 20 min. After the addition of SDS (0.8% final concentration), samples were resolved on a 0.8% agarose gel at 23 V in 0.5x TBE buffer (pH 8.2) for 16 h. After electrophoresis, the gels were stained with ethidium bromide (1-2 µg/ml) visualized, and photographed under UV illumination using Syngene gel documentation system, UK.

2.21. Strand exchange assay of Ssb variants with cognate Deinococcal RecA and noncognate *E. coli* RecA.

The assay was performed as described earlier [21-23]. Briefly, the reaction mixture contained 10 µM Øx174 ssDNA and 10 µM Øx174 linear double stranded DNA (dsDNA) in 20 mM Tris acetate buffer, pH 7.9, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothretiol, ATP (3 mM) and ATP regeneration system (12 mM phosphocreatine and 10 units/ml of phosphocreatine kinase). In one set of reactions (SS-DS mode) Øx174 ssDNA was first incubated with 3 µM Deinococcal RecA or *E. coli* RecA in reaction buffer containing 3 mM ATP for 10 min at 37°C. Individual variants of Ssb proteins or their

combination were then added and incubated for another 10 min. Finally, reaction was initiated by addition of linear Øx174 dsDNA. A parallel set of reactions (DS-SS mode) [114] was also conducted in which Deinococcal RecA was first incubated with linear ØX174 DS DNA for 40 min, then ØX174 ssDNA was added, and after 5 min of incubation individual Ssb variants were added. In both modes, the reaction was terminated after 2 h by adding SDS to a final concentration of 1.25% and the reaction products were resolved on a 0.8% agarose gel at 23 V in 0.5x TBE buffer (pH 8.2) for 16 h. After electrophoresis, the gel was stained with ethdium bromide and the products were visualized and photographed under UV illumination using Syngene Gel Documentation System, U. K.

2.22. Immunodetection of stress responsive Ssb and RecA expression.

2.22.1. Treatment with various stresses.

Wild type *D. radiodurans* cells were grown overnight at 32ºC to OD_{600nm} of 4-5/ml and subjected to one of the following stress treatments: (a) 2-8 kGy of γ-radiation from two different ^{60}Co source (GC220, Atomic Energy of Canada Limited, Canada, dose rate 0.36 kGy hr^{-1}) or (GC5000, BRIT, India, dose rate 7.2 kGy hr^{-1}) (b) 20 µg ml^{-1} of mitomycin-C (Sigma, St. Louis, USA) for 15 min at 32°C (c) 50 or 100 mM of hydrogen peroxide (Ranchem, New Delhi, India) for 1 h at 32°C, or (d) 2.5 or 5 kJ m^{-2} of UV (Philips, India dose rate 5 J m^{-2} s^{-1}). UV stress was applied to 4 ml culture spread in a 9 cm diameter sterile petri plate, to form <1 mm thin layer and to minimize absorption by the TGY medium. For desiccation stress, overnight grown *D. radiodurans* cells were filtered and subjected to 1, 2 or 6 weeks of desiccation using fused calcium chloride in desiccators stored at 23°C. Following stress treatment, cells were washed three times with fresh TGY medium,

inoculated in TGY at a final OD_{600nm} of 0.5 and allowed to recover. Periodically samples were removed for western blots and colony counts.

2.22.2. Quantitation of Ssb and RecA protein levels by immunodetection under various stresses.

For detection and quantitation of Ssb and RecA protein levels, proteins were extracted from wild type *D. radiodurans* after indicated time of post stress recovery. The samples were removed at indicated time points and Deinococcal cells were disrupted by heat treatment at 100°C in presence of cracking buffer (Table 2.2) for 10 mins. The clarified cellular extracts were quantitated by Lowry's method of protein estimation. Protein extracts (30-100 µg) from control or stressed samples were resolved by 12% SDS-PAGE and electroblotted on nitrocellulose membrane. Primary antibody used was anti-*D. radiodurans* Ssb antibody (anti-DrSSB) at 1:200 dilution or anti-DrRecA antibody at 1:500 dilution. Blots were incubated with primary antibody overnight, followed by two washes with TS buffer. Secondary antibody anti-rabbitIgG (coupled to alkaline phosphatase) (Sigma, St. Louis, USA) was used at 1:10,000 dilution and incubated with blot for 1.5-2 h. The blot was subsequently again washed twice with TS buffer and developed using substrate nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Roche Biochemicals, Gmbh, Germany). The bands so obtained were quantitated by Gel Quant software (Biosystematica, UK). All experiments were repeated at least three times and data on enhancement in Ssb levels were plotted with standard deviation.

2.22.3. Transcriptional induction of *ssb* gene expression.

For assessment of transcriptional activation of *ssb* expression, overnight grown wild type *D. radiodurans* cells (OD_{600nm}=5 ml^{-1}) were divided into three sets. To one set rifampicin (50 µg ml^{-1}) was added prior to γ-radiation of 7 kGy (dose rate=0.36 kGy hr^{-1}) and washed off before PIR, to the second set rifampicin was added after irradiation but before PIR of cells. The samples were withdrawn at indicated time points from both the sets. The third set had Deinococcal cells in TGY medium with rifampicin (50 µg ml^{-1}) which was irradiated, rifampicin washed off after irradiation and fresh rifampicin added to cells during PIR of irradiated cells. The sample from this additional treatment was withdrawn only at 3 h of post irradiation recovery. All treatments had their corresponding unirradiated controls. The samples were processed and Ssb protein levels immunodetected as described above.

2.23. Assessment of role of RDRM in *ssb* gene expression.

The assessment of role of RDRM sequences in *ssb* gene expression was made by constructing various deletions of RDRM sequences from upstream of Deinococcal *ssb* gene, and fusion to a nonspecific acid phosphatase encoding reporter gene *phoN*. For construction of pSN4 plasmid, 351bps upstream region of *ssb* ORF was amplified and cloned in pRN1 (promoter less vector having *phoN* as reporter gene). pSN3 plasmid was constructed by cloning 132 bps upstream region of *ssb* ORF in pRN1, pSN2 plasmid was constructed by cloning 102 bps upstream region of *ssb* ORF in pRN1. pSN4 plasmid resulted in inclusion of both RDRM sequences, pSN3 plasmid included only one reported RDRM sequence while pSN2 acted as a control without any of the complete RDRM elements. The constructs generated were transformed in *Deinococcus radiodurans* and subjected to different stresses

as described previously. The *ssb* promoter activity was assessed by zymogram analysis of Deinococcal cells at 3h post stress recovery. Proteins were extracted from cells in non-reducing buffer, estimated by lowry's method and resolved by 12% SDS-PAGE. After the electrophoresis, the acrylamide gel was rinsed briefly with distil water to remove most of sodium dodecyl sulfate and further gel proteins were renatured by giving two washes of 1% Triton X-100 in 100 mM acetate buffer (pH 5.0) for 20 min each, followed by a wash with 100 mM acetate buffer without Triton X-100. PhoN activity bands were developed by using NBT/BCIP as chromogenic substrate in 100 mM acetate buffer pH 5.0. The development assay was stopped by rinsing the gel in distilled water after the appearance of bands.

2.24. Source of materials.

Restriction enzymes, vectors (pTWIN1 & pMBX10), DNA (ØX virion, ØX dsDNA and M13MP18 dsDNA), *E. coli* Topoisomerase I, *E. coli* RecA and chitin beads were obtained from New England Biolabs, UK. T4 DNA ligase, Q-sepharose, Sephadex 50 columns, terminal transferase, digoxigenin (DIG) labeled ddUTP, nylon membrane, anti-DIG antibody, nitrocellulose membrane and NBT-BCIP solution were obtained from Roche Life Sciences, UK. PCR extraction kit, Gel extraction kit and Ni-NTA matrix were procured from Qiagen, Germany. Sodium dodecyl sulphate (SDS), 2-mercaptoethanol (2-ME), hydrogen peroxide, Lowry's reagent, rifampicin, carbenicillin, ethidium bromide, IPTG, ATP, phosphocreatine, phosphocreatine kinase and *E. coli* Ssb protein were obtained from *Sigma*-Aldrich *India*. Bacterial growth medium components were from BD and Co. (Becton, Dickinson and Company), India. Oligo dT50 was obtained from MWG biotech,

India. Genomic DNA isolation kit was obtained from Hi-media laboratories, India. Inorganic salts were obtained from Qualigens, India.

Chapter 3
Results and Discussion

Deinococcus-Thermus group of bacteria encode Ssb proteins which are different from the prototype *Escherichia coli Ssb.* They contain two OB folds and function as homodimers [14], in contrast to the homotetrameric *E. coli* Ssb that harbours a single OB fold or heterotrimeric eukaryotic RPA (Replication protein A) [10, 15]. Transcriptomic and proteomic analyses of Deinococcal cells following gamma radiation stress have revealed up-regulation of several DNA repair proteins [50, 165] . Among these, Ssb has been shown to be the most abundant protein [163], which together with gyrase, RecA, topoisomerase I and RecQ helicase, is recruited to the Deinococcal nucleoid [156]. Biochemical analyses have revealed that Deinococcal Ssb displays salt-independent weak ssDNA binding activity and displaces shorter strand of duplex DNA than *E. coli* Ssb [17, 18].

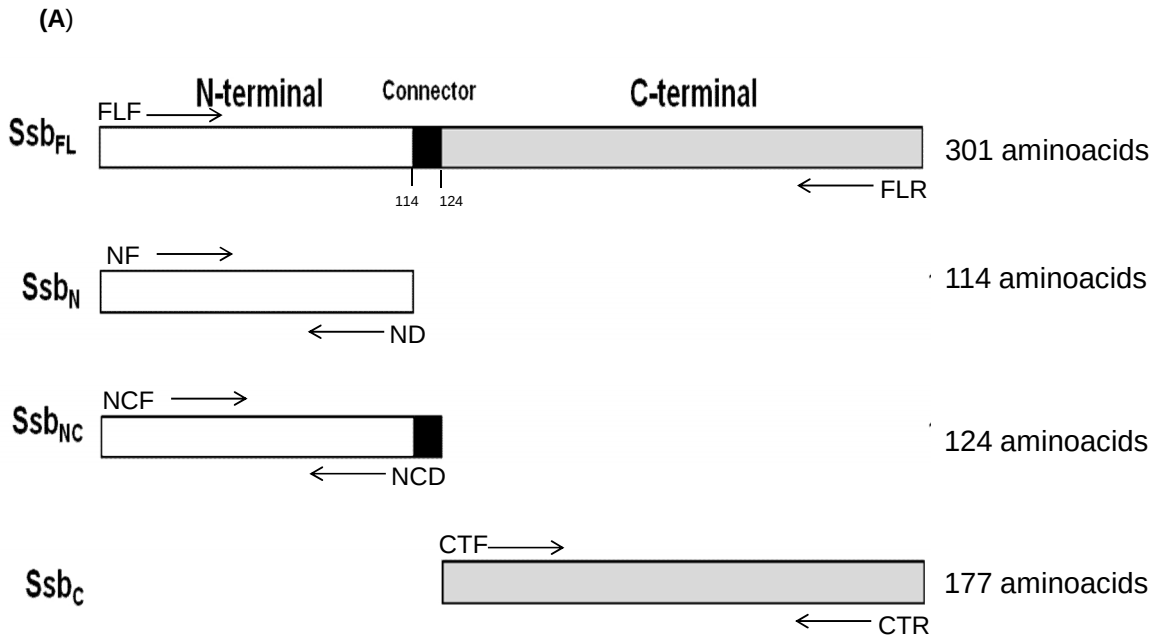
Bioinformatic analyses have indicated differences between the two OB folds [14]. Crystal structure has confirmed a structural asymmetry between the two domains and suggested that each OB fold may have evolved for a specialized role. It also showed that Deinococcal Ssb dimer formation occurred using an extensive surface area formed by N-terminal and the connector region. The interface had hydrogen, ionic and van der Waals interaction and was quite different from *E. coli* Ssb tetramer formation which involved L_{45} loop mediated interaction [7, 19, 20].

In this study, a structure-based functional analysis of *Deinococcus radiodurans* Ssb was undertaken. The nucleotide sequences corresponding to the full length protein (Ssb_{FL}, amino acids 1-301), N-terminal OB domain (Ssb_N, amino acids 1-114), N-terminal OB domain with connector ($Ssb_{NC,}$ amino acids 1-124) and C-terminal OB domain (Ssb_C, amino acids 125-301) were cloned, over-expressed and purified from *E. coli*. Protein-DNA and protein-protein interaction assays revealed differential ssDNA binding and oligomerization patterns

of different domains as well as demonstrated their physical and functional interaction to enhance ssDNA binding between N-terminal and C-terminal domains. Role of individual domains vis-à-vis the full length Ssb in carrying out more complex reactions was also investigated. Expression profiling of Ssb protein revealed its transcriptional activation upon DNA damage mediated through two RDRM sequences present upstream [164].

3.1.1. Organization of Deinococcal *ssb* gene.

The *ssb* gene of *Deinococcus radiodurans* is located 119 bps down stream of the *rpsF* (30S ribosomal subunit protein S6) gene and is followed by the *rpsR* (30S ribosomal subunit protein S18) gene on chromosome I. The various Ssb variants generated for this study and location of the primers used for cloning different Ssb variants is shown in Fig 3.1.



(B)

FLF →

TCTAGTTATTTTGCCAGCTACCCAAGGAGAATTTGTT**ATG**GCCCGAGGCATGAACCACGTCTACCTGATCGGC

NCF,NF →

GCACTCGCCCGTGATCCCGAACTTCGCTACACCGGCAACGGCATGGCCGTTTTCGAAGCCACCGTCGCAGG
CGAAGACCGAGTGATCGGCAATGATGGCCGTGAACGCAATCTGCCCTGGTACCACCGCGTGTCGATTCTGG
GCAAGCCCGCCGAATGGCAGGCCGAGCGCAACCTCAAGGGCGGCGACGCCGTGGTGGTTGAAGGTACCC
TGGAATACCGTCAGTGGGAAGCGCCCGAAGGCGGCAAACGCAGCGCAGTCAACGTCAAAGCCCTCCGCAT

CTF →

GGAACAGCTCGGCACCCAGCCCGAACTGATTCAGGACGCTGGCGGCGGCGTGCGCATGAGCGGCGCGAT

← ND ← NCD

GAATGAAGTCCTGGTCCTCGGCAACGTGACCCGTGACCCCGAGATTCGCTACACCCCTGCGGGTGACGCTG
TGCTGAGCCTGTCGATTGCGGTCAACGAGAACTACCAGGACCGTCAGGGCCAGCGCCAGGAAAAGGTTCAC
TATATCGACGCCACGCTCTGGCGCGACCTCGCCGAGAACATGAAGGAGCTCCGTAAGGGCGACCCGGTCAT
GATCATGGGCAGGCTCGTCAACGAGGGCTGGACCGATAAAGACGGCAACAAGCGCAACAGCACAAGAGTAG
AGGCGACGCGAGTCGAAGCCCTTGCCCGAGGCGCAGGCAATGCCAATTCGGGCTACGCCGCAGCCACCCC
TGCCGCACCTCGCACGCAGACCGCGAGCAGTGCCGCACGCCCGACGTCTGGCGGCTACCAGAGCCAGCC
CAGCCGCGCGGCGAACACGGGGAGCCGTTCGGGGGGCCTAGATATTGATCAAGGTCTCGACGATTTTCCGC
CGGAAGAAGACGACCTGCCCTTT**TAA**TGCAGGCCCTGGGGGCCTAGCGCCCCCGGCTTCCGGCCACACCA

← CTR

AGGACACCCAACATGACCCAGCAAGGAAACAGCGG

← FLR

Fig. 3.1. Organization of *Deinococcal radiodurans ssb* gene. (A) Schematic representation of Deinococcal Ssb protein and its variants constructed in this study. (B) Nucleotide sequence of *ssb* gene showing the position of N-terminal region (red), connector region (green) and C-terminal region (blue). Start and stop codon are shown in bold. Primer sequences used for cloning different variants are underlined.

Sequence similarity of Deinococcal Ssb was compared among its two OB fold domains and also with *E. coli* Ssb individually. The analysis showed that Ssb_{NC} shared 33% identity and 51% similarity with Ssb_C and 38% identity and 50% similarity with *E. coli* Ssb (BlastP analysis, Figure 3.2). Ssb_C also shared 39% identity and 64% similarity with *E. coli* Ssb protein [14] and was more similar to *E. coli* Ssb than to Ssb_{NC}.

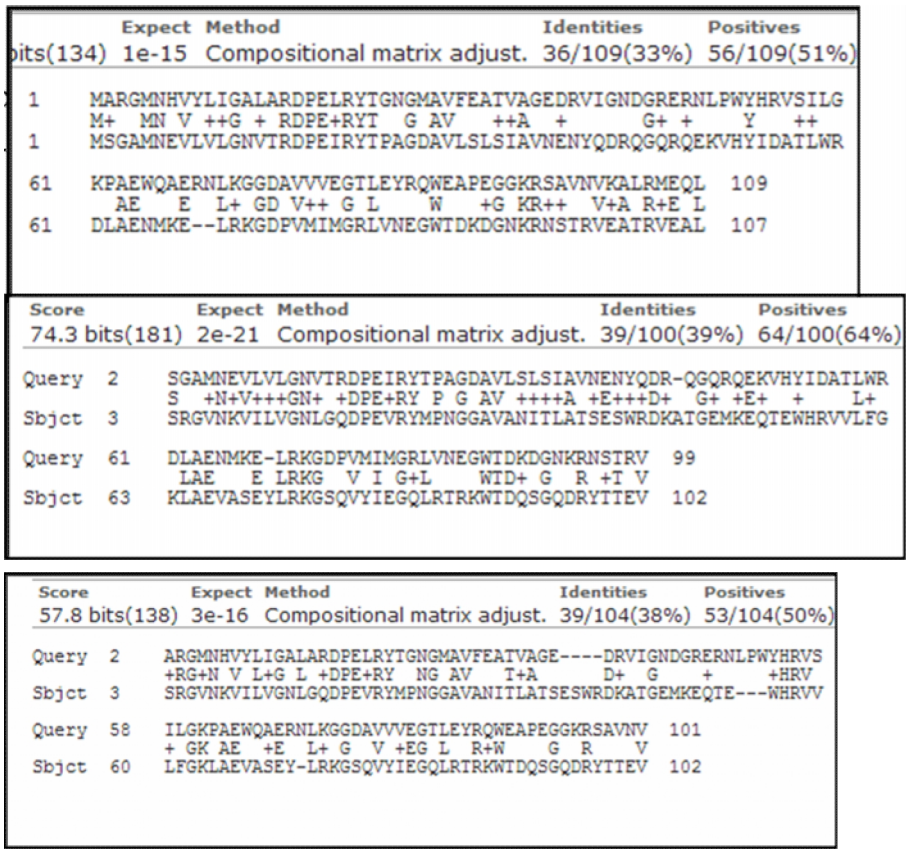


Fig. 3.2. BlastP comparison analysis of various Deinococcal Ssb variants. Comparison of Ssb_C (CT) with Ssb_{NC} (NC) as well as comparison of Ssb_C with *E. coli* Ssb (EC) and comparison of Ssb_{NC} with *E. coli* Ssb was done by using BlastP algorithm (www. http://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.1.2. Over-expression and purification of full length Ssb (Ssb_{FL}).

The full length *ssb* gene was PCR amplified using specific primers (FLF and FLR, Table 2.1). The product was digested with *Bam*HI and ligated to pUC19 at *Bam*HI site to obtain the plasmid pUC19Ssb_{FL}. The insert from pUC19Ssb_{FL} was released by digesting it with *Nde*I and *Bam*HI and ligated to pET16b at identical sites (Fig 3.3 A and B). The construct pET16b (Ssb_{FL}) thus generated was transformed into *E. coli* BL-21(pLysS) cells. Transformants were grown in LB medium, supplemented with carbenicillin, at 37°C and induced by IPTG. The expression kinetics of the Ssb_{FL} protein in cell free extracts was monitored over a 6 h period by 12% SDS PAGE. Maximum overexpression of Ssb_{FL} was observed after 4 h of induction in soluble fraction (Fig 3.3 C). The His tagged Ssb_{FL} protein was first purified by Ni-NTA affinity purification protocol and eluted by an imidazole gradient (Fig 3.3 D). Minor contaminant proteins seen were further removed by passing the purified fractions through Q-sepharose. The column was eluted by a NaCl gradient (Fig 3.3 E). Protein fractions were checked subsequently for any nuclease contamination and fractions free of any exo or endonuclease activity were pooled together. The purified protein (Fig 3.3 F) was dialyzed overnight in Ssb storage buffer, snap frozen in liquid nitrogen and stored at -70°C until further use.

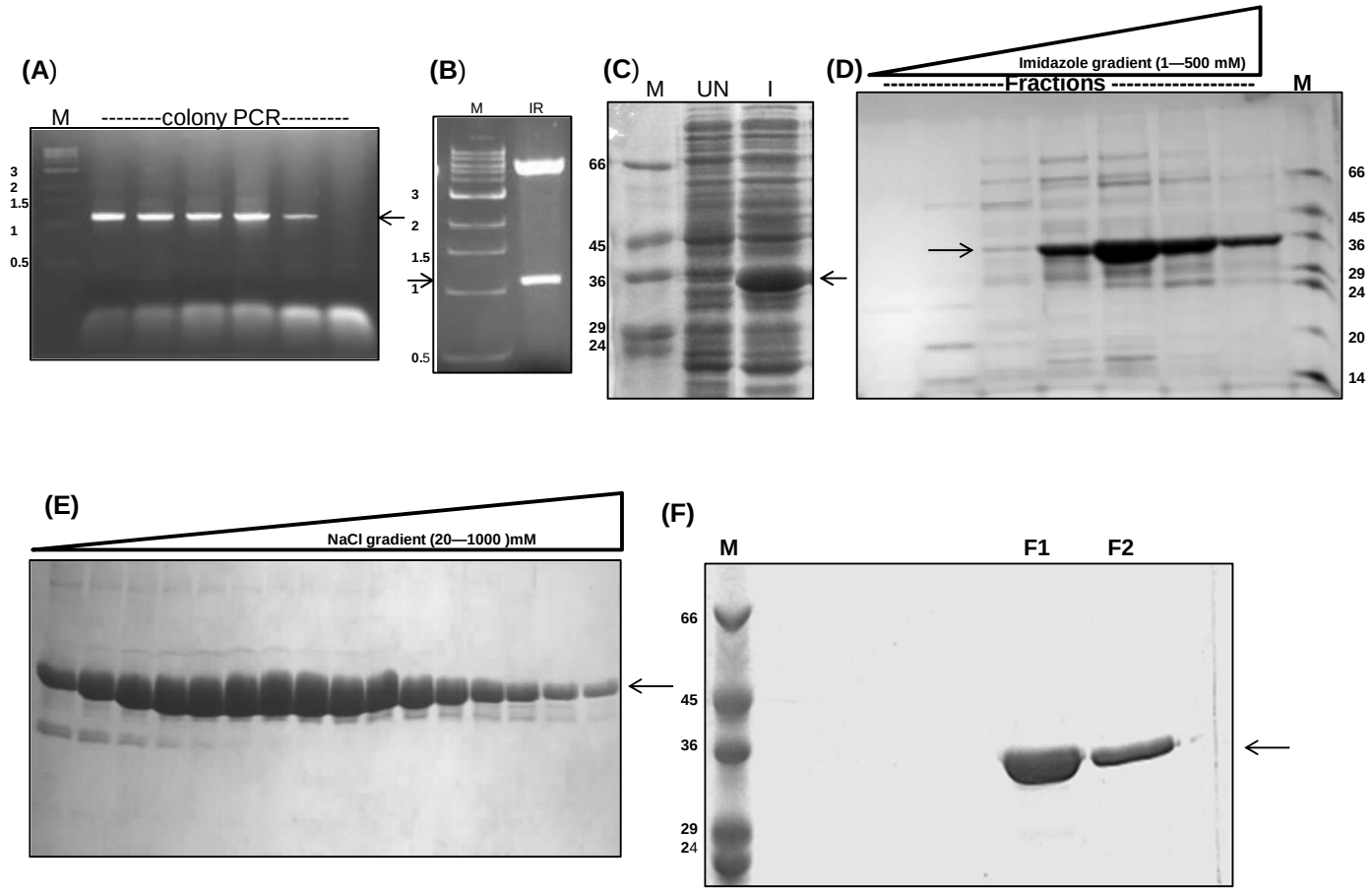


Fig. 3.3. Purification of Ssb_{FL}. (A) Colony PCR of 6 *E. coli* colonies transformed with pET16b (Ssb_{FL}) plasmid, using FLF and FLR primer. The 1 kb DNA ladder is shown beside the figure. The correct size band is shown by arrow. (B) pET16b (Ssb_{FL}) recombinant plasmid from a transformant, confirmed by colony PCR was subjected to restriction analysis by *Nde*/*Bam*HI and *ssb* gene insert was released [IR lane], lane M contained 1 kb DNA ladder. (C) The cellular proteins of *E. coli* BL-21 cells over-expressing Ssb_{FL} resolved by 12% SDS-PAGE. UN and I indicate extracts made before and after IPTG induction for 4 h. Lane M contained SDS-7 protein standard in kDa. (D) The protein profile of various fractions, obtained after Ni-NTA column purification, and resolved by 12% SDS-PAGE. Elution was done by immidazole gradient (1-500 mM) and 10 µl of each fraction loaded onto the gel. Lane M contained SDS-7 protein standard in kDa. (E) The protein profile of various fractions obtained after Q-sepharose column purification, resolved by 12% SDS-PAGE. Elution was done by NaCl gradient (20-1000 mM) and 10 µl of each fraction loaded onto the gel. (F) SDS PAGE profile of purified fraction of Ssb_{FL} devoid of any nuclease contamination. Two different fractions (F1 and F2) were made. Lane M contained SDS-7 protein standard in kDa.

3.1.3. Over-expression and purification of C-terminal region of Deinococcal Ssb (Ssb_C).

The C-terminal region of *ssb* gene (Ssb_C) was PCR amplified using specific primers CTF and CTD (Table 2.1), restriction digested with *Nde*I/*Bam*HI and ligated to identical restriction sites in pET16b (Fig 3.4A and B). The construct pET16b (Ssb_C), thus generated, was transformed into *E. coli* BL-21(pLysS) cells. Transformant *E. coli* BL-21 [pET16b(Ssb_C)] cells were grown in LB medium, supplemented with carbenicillin, at 37°C and induced by IPTG. The expression kinetics of the Ssb_C protein (from cell free extracts) was monitored over 6 h period by 12% SDS-PAGE. Maximum overexpression was observed after 4 h of induction in soluble fraction for Ssb_C protein (Fig 3.4C). The His tagged Ssb_C proteins was first purified by Ni-NTA affinity purification protocol and eluted by an imidazole gradient (Fig 3.4D). Minor contaminant proteins seen were further removed by passing the purified fractions through Q-sepharose. The column was eluted by a NaCl gradient (Fig 3.4E). Protein fractions were checked subsequently for any nuclease contamination and fractions free of any exo or endonuclease were pooled together. The purified protein was dialyzed overnight in Ssb storage buffer, snap frozen in liquid nitrogen and stored at -70°C until further use (Fig 3.4F).

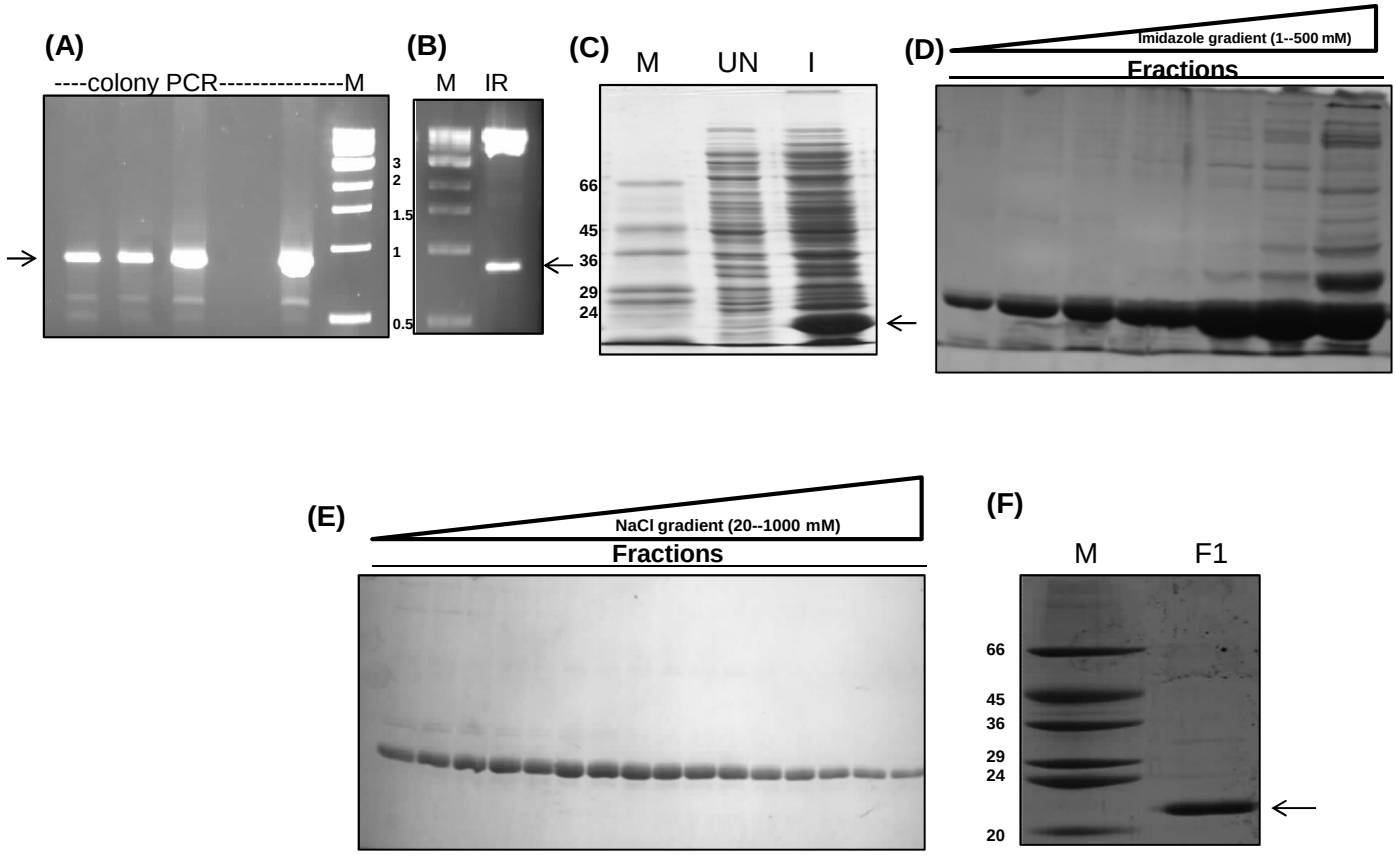


Fig. 3.4. Purification of $Ssb_{C.}$ (A) Colony PCR of *E. coli* transformed with pET16b (Ssb_C) plasmid using CTF and CTD primer. The 1 kb DNA ladder is shown beside the figure. The correct size band is shown by arrow. (B) pET16b (Ssb_C) recombinant plasmid from a transformant, confirmed by colony PCR was subjected to restriction analysis by *Nde*/*Bam*HI and Ssb_C insert was released [IR lane], lane M contained 1 kb DNA ladder. (C) The cellular proteins of *E. coli* BL-21 cells over-expressing Ssb_C resolved by 12% SDS-PAGE. UN and I indicate extracts made before and after IPTG induction for 4 h. Lane M contained SDS-7 protein standard in kDa. (D) The protein profile of various fractions obtained after Ni-NTA column purification resolved by 12% SDS-PAGE. Elution was done by imidazole gradient (1-500 mM) and 10 µl of each fraction loaded onto the gel. (E) The protein profile of various fractions obtained after Q-sepharose column purification, resolved by 12% SDS-PAGE. Elution was done by NaCl gradient (20-1000 mM) and 10 µl of each fraction loaded onto the gel. (F) SDS PAGE profile of purified fraction (estimated to be ~95% by densitometry) of Ssb_C devoid of any nuclease contamination. One fraction (F1) was collected. Lane M contained SDS-7 protein standard in kDa.

3.1.4. Over-expression and purification of N-terminal with connector region of Deinococcal Ssb (Ssb_{NC}).

Purification of His-tagged Ssb_N or Ssb_{NC} by Ni-NTA affinity chromatography proved to be rather difficult, since they bound Ni-NTA matrix poorly. The genes were cloned independently with N or C-terminal His tag but in both cases, the tagged proteins still did not bind to Ni-NTA column. Attempts were also made to tag the protein with maltose binding protein (MBP), which can bind to amylose resin and tagged protein can be subsequently eluted by maltose gradient. The tag can subsequently be cleaved by factor Xa protease. However, there were problems with protease cleavage and target proteins could not be purified, even after several attempts and modifications of protocol.

The proteins were finally affinity purified, using an intein tag having a chitin binding domain. Two vectors, pTwin1 or pMXB10, were used for the purpose (Table 2.1). These vectors have N/C terminal intein as tag, which can be removed by pH or temperature change in case of pTwin vector and by addition of DTT in case of pMXB10 vector.

DNA of N-terminal with connector Ssb (Ssb_{NC}) was amplified from genomic DNA using specific primers NCF and NCD (Table 2.1), restriction digested with *Eco*RI/ *Bam*HI and ligated to pTwin1 vector at identical restriction sites (Fig. 3.5A and B). In pTwin1 vector the tag is present at the N-terminal of protein which is subsequently removed by on column cleavage. The construct pTwin(Ssb_{NC}), thus generated, was transformed in *E. coli* ER2566 strain. Transformant *E. coli* ERSsb_{NC} cells were grown in LB medium, supplemented with carbenicillin, at 20°C and induced by IPTG (Fig. 3.5C). The clarified cell free extract containing tagged Ssb_{NC} (in 20 mM Tris-HCl pH 8.5, 0.5 M NaCl) was loaded on chitin beads.

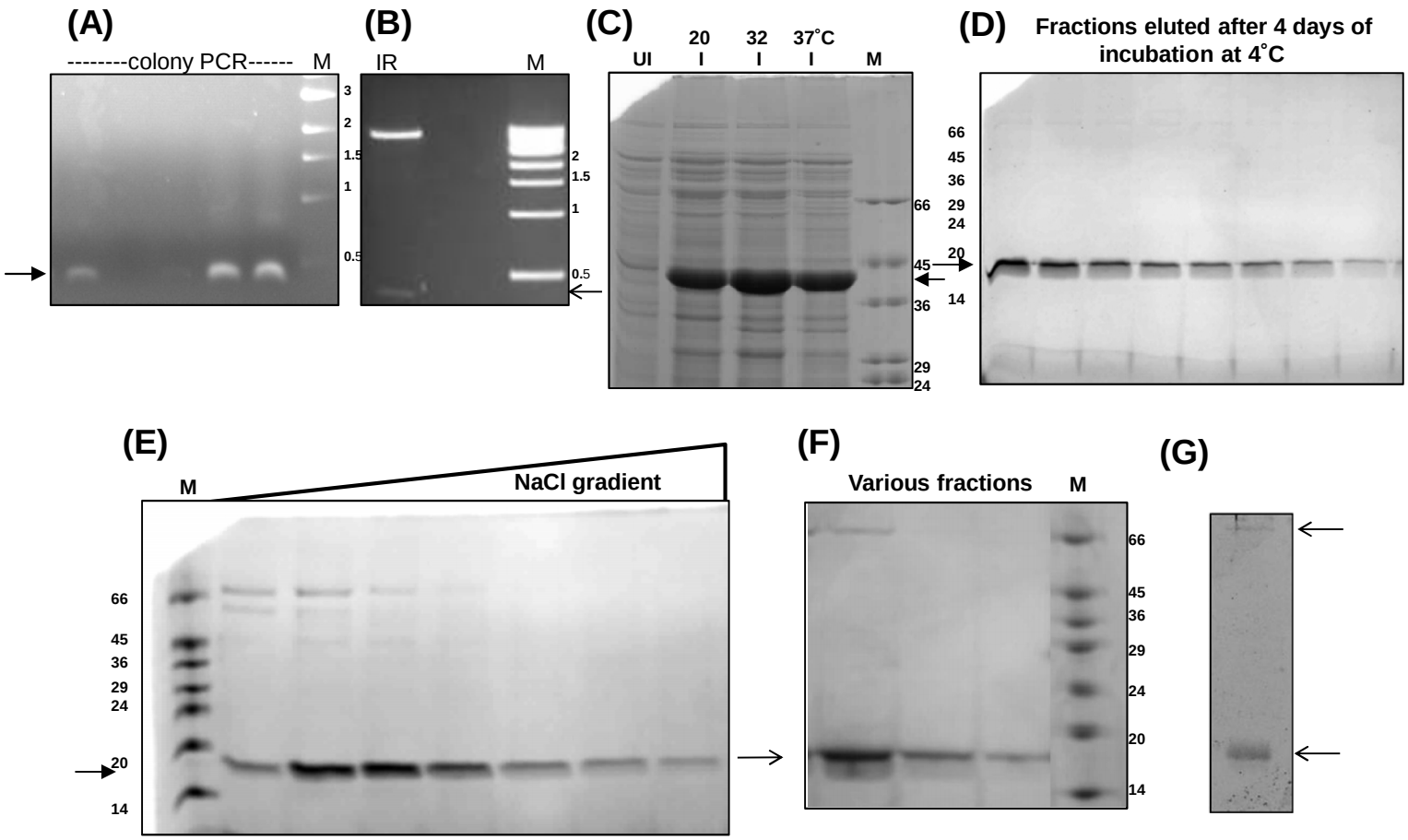


Fig. 3.5. Purification of Ssb_{NC}. (A) Colony PCR of *E. coli* transformed with pTwin (Ssb_{NC}) plasmid, using NCF and NCD primer. The 1 kb DNA ladder is shown beside the figure. The correct sized band is shown by arrow. (B) pTwin (Ssb_{NC}) recombinant plasmid, from a transformant confirmed by colony PCR, was subjected to restriction analysis by *Eco*RI/ *Bam*HI and N-terminal with connector region (Ssb_{NC}) insert was released as shown by arrow. The 1 kb DNA ladder is shown beside the figure. (C) The cellular proteins of *E. coli* ER2566 cells over-expressing intein tagged Ssb_{NC} (arrowed) at different temperatures were resolved by 14% SDS PAGE. Lane M contained SDS-7 protein standard in kDa. (D) Protein profile of various fractions obtained after Chitin beads column purification. Elution was done after 4 days of incubation of column at 20mM phosphate buffer pH 6.0 and 10 µl of the each eluted fraction was resolved by 14% SDS PAGE. The arrow shows Ssb_{NC} protein. (E) Protein profile of various fractions obtained after Q-sepharose column purification resolved by 14% SDS PAGE. Elution was done by NaCl gradient and 10 µl of each eluted fraction was resolved by 14% SDS PAGE. Lane M contained SDS-7 protein standard in kDa. (F) Purified Ssb_{NC} (arrowed) resolved by 14% SDS PAGE. Lane M contained SDS-7 protein standard in kDa. (G) Purified Ssb_{NC} (arrowed) immunodetected using anti-Ssb_{FL} antibody.

On-column cleavage of Ssb_{NC} protein was done by equilibrating the column with sodium phosphate buffer pH 6.0 containing 0.5M NaCl and incubating it at 4°C for 4 days. Final elution of columns was simply done by passing buffer with which on column cleavage was initiated. The fractions so obtained were analyzed by 14% SDS-PAGE (Fig. 3.5D). Minor contaminant proteins seen were further removed by passing the purified pooled fractions through Q-sepharose. The column was eluted by a NaCl gradient (Fig 3.5E). Protein fractions were checked subsequently for any nuclease contamination and fractions free of any exo or endonuclease activity were pooled together. The purified Ssb_{NC} protein was dialyzed overnight in Ssb storage buffer, snap frozen in liquid nitrogen and stored at -70°C until further use (Fig 3.5F).

3.1.5. Over-expression and purification of N-terminal region of Deinococcal Ssb (Ssb_N).

DNA of N-terminal without connector (Ssb_N) was amplified using specific primer NF and ND (Table 2.1), restriction digested with *Nde*I and *Xho*I and ligated to identical restriction sites in pMXB10 vector (Fig. 3.6A and B). In pMXB10 vector the tag is at C-terminal region of protein, which is subsequently removed by on column cleavage. The constructs thus generated, were transformed in *E. coli* 2566 strain. Transformant *E. coli* $ERSsb_N$ cells were grown in LB medium, supplemented with carbenicillin at 20°C and induced by IPTG (Fig. 3.6C). The clarified cell free extract containing tagged Ssb_N (in 20 mM Tris-HCl pH 8.5, 0.5 M NaCl) was loaded on chitin beads.

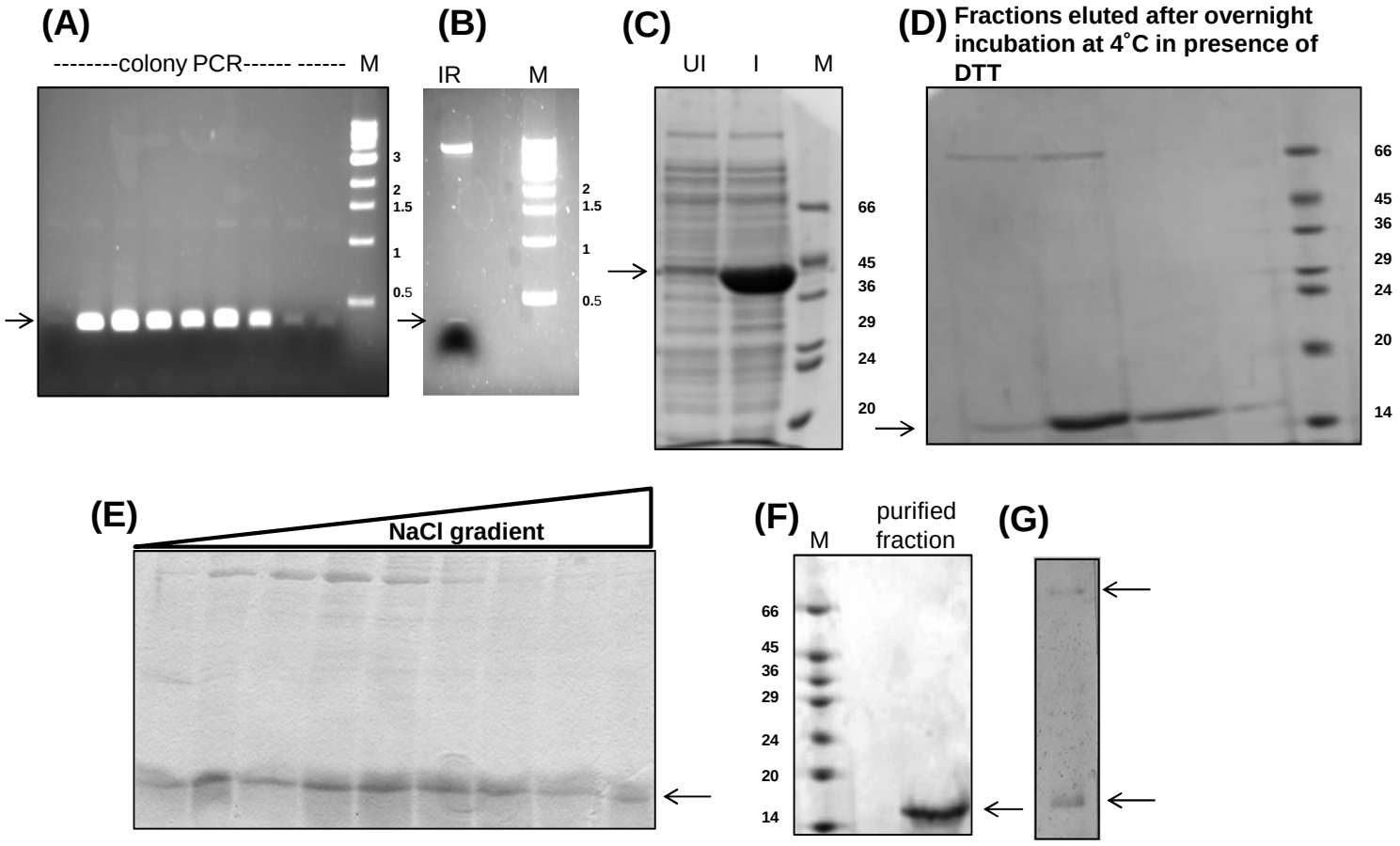


Fig. 3.6. Purification of $Ssb_{N:}$ (A) Colony PCR of *E. coli* transformed with pMXB10(Ssb_N) plasmid, using NF and ND primers. The 1 kb DNA ladder is shown beside the figure. The correct sized band is shown by arrow. (B) pMXB10(Ssb_N) recombinant plasmid, from a transformant confirmed by colony PCR, was subjected to restriction analysis by *Nde*I/ *Xho*I and N-terminal region (Ssb_N) insert was released as shown by arrow. (C) The cellular proteins of *E. coli* ER2566 cells over-expressing intein tagged Ssb_N (arrowed) at 20°C temperature was resolved by 14% SDS PAGE. Lane M contained SDS-7 protein standard in kDa. (D) Protein profile of various fractions obtained after Chitin beads column purification. Elution was done after overnight incubation of column with DTT at 4°C and 10 µl of each eluted fraction was resolved by 14% SDS PAGE. Lane M contained SDS-7 protein standard in kDa. (E) Protein profile of various fractions obtained after Q-sepharose column purification, resolved by 14% SDS PAGE. Elution was done by NaCl gradient and 10 µl of each eluted fraction was resolved by 14% SDS PAGE. The arrow shows Ssb_N protein. (F) Purified Ssb_N (arrowed) resolved by 14% SDS PAGE. Lane M contained SDS-7 protein standard in kDa. (G) Purified Ssb_N (arrowed) immunodetected using anti-Ssb_{FL} antibody.

On column cleavage was done by quickly equilibrating the column with 20mM Tris, 0.5M NaCl, pH 8.5 buffer containing 10mM DTT and incubated overnight at 4°C. Final elution of columns was simply done by passing buffers with which on column cleavage was initiated without DTT. The fractions so obtained were analyzed by SDS-PAGE to confirm the purity of elutant (Fig. 3.6D). Minor contaminant proteins seen were further removed by passing the purified pooled fractions through Q-sepharose. The column was eluted by a NaCl gradient (Fig. 3.6E). Protein fractions were checked subsequently for any nuclease contamination and fractions free of any exo or endonuclease activity were pooled together. The purified Ssb_N protein was dialyzed overnight in Ssb storage buffer, snap frozen in liquid nitrogen and stored at -70°C until further use (Fig. 3.6F).

The identity of Ssb_{NC} and Ssb_N was confirmed by immunodetection by using antibody against Ssb_{FL} (Fig 3.5, 3.6 G) as well as by mass spectrometery. The data of mass spectrometery is shown below.

Table 3.1 Mass spectrometry data of Ssb_N and Ssb_{NC}.

Protein	Mascot score	Peptides matched	Sequence coverage
Ssb_N	64	9	54%
Ssb_{NC}	77	10	55%

3.1.6. Specificity of anti-SsbC antibody.

The N and C-terminal domain of Ssb were markedly different in overall amino acid composition and an antibody raised against the C-terminal region (anti-$DrSsb_C$) did not recognize the N-terminal domain of Ssb in immunodetection (Figure 3.7). Thus, the epitopes formed by Ssb_C were markedly different from those carried by N terminal domain.

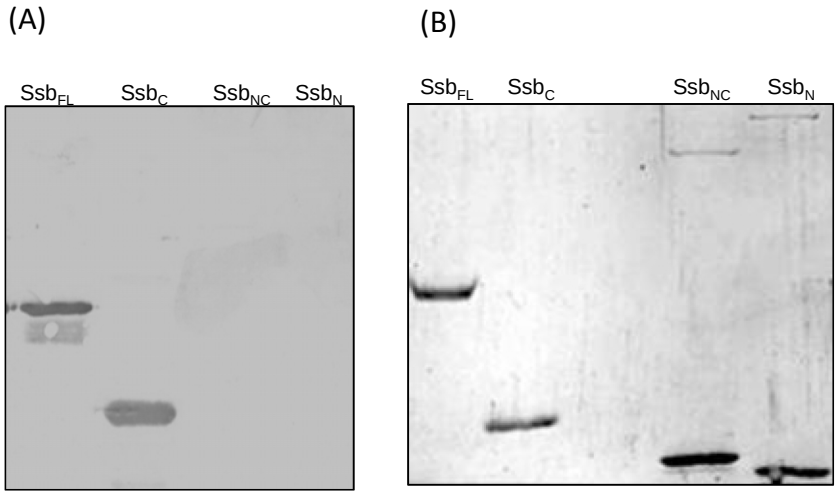


Fig. 3.7. Specificity of anti-Ssb_C antibody. (A) About 1-2 µg of specified purified proteins were resolved by 14% SDS page, blotted onto nitrocellulose membrane, probed using anti-Ssb_C antibody and developed using NBT-BCIP substrate. (B) CBB stained gel corresponding to western blot shown in (A).

3.1.7. Large scale purification of RecA of *Deinococcus radiodurans* without any tag.

The Deinococcal RecA protein was purified by 3 different methods involving various vectors but those used subsequently for various assays was purified as described by Kim et.al [169] with some modification. RecA gene was PCR amplified using specific primers RF and RD (Table 2.1), restriction digested with *Nco*I and *Bam*HI and ligated to pET16b at identical sites (Fig. 3.8 A and B).

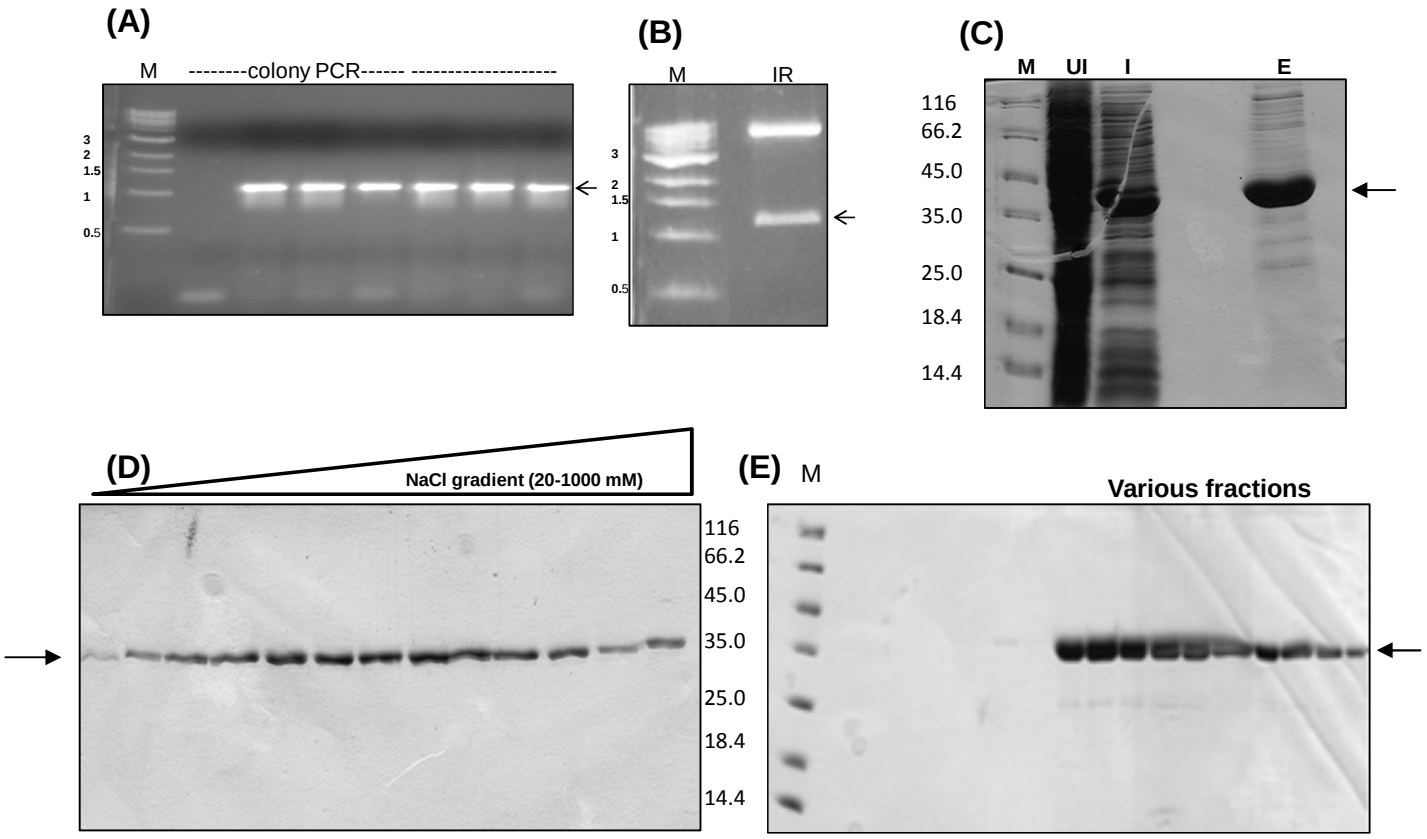


Fig. 3.8. Purification of Deinococcal RecA protein. (A) Colony PCR of *E. coli* transformed with pET16b(RecA) plasmid using RF and RD primer. The 1 kb DNA ladder is shown beside the figure. The correct sized band is shown by arrow. (B) pET16b(RecA) recombinant plasmid from a transformant, confirmed by colony PCR was subjected to restriction analysis by *Nco*I/ *Bam*HI and *rec*A gene insert was released. (C) SDS PAGE profile showing various stages of purification of Deinococcal RecA, over-expressed in *E. coli* STL2669 cells. Protein standard marker in kDa, un induced and IPTG induced cell extract are shown. Lane E depicts the purity of protein after ammonium sulphate treatment. (D) SDS PAGE profile of various fractions obtained after Q-sepharose column purification. Elution was done by NaCl gradient (20-1000 mM) and 10 µl of each fraction loaded onto the gel. (E) SDS PAGE profile of purified fraction of RecA devoid of any nuclease contamination. M lane depicts protein standard marker in kDa.

The construct pET16b(RecA), thus generated, was transformed into *E. coli* STL2669 overexpression strain. Transformant *E. coli* STLRecA cells were grown in LB medium supplemented with carbenicillin, at 37°C and induced by IPTG. A cell free extract was obtained after sonication and RecA protein was precipitated by adding Polymin-P (pH 7.9). Subsequently the protein was eluted from pellet using ammonium sulphate as described in materials and methods (Fig 3.8C). Minor contaminant proteins seen were further removed by passing the purified fractions through Q-sepharose. The column was eluted by a NaCl gradient (Fig 3.8D). The protein fractions, free of any exo and endonuclease activity were dialyzed overnight in storage buffer (20mM Tris pH7.5, 0.1mM EDTA, 1mM DTT and 10% v/v glycerol) then aliquoted, snap-frozen and stored at -70°C (Fig 3.8E).

3.1.8. Ascertaining lack of nuclease contamination in purified Deinococcal Ssb_{FL}, Ssb_{C}, Ssb_{NC}, Ssb_{N} and RecA proteins.

As all the candidate proteins use DNA as substrate, for their characterization it was essential to check for any nuclease contamination. Standard nuclease assay, using M13 as substrate was adopted for this purpose. M13 replicative form or M13 single stranded DNA were used as substrate and reaction was carried out in the presence of magnesium chloride and ATP. Many of the potent nucleases, which are not easily seen as contaminating bands on SDS-PAGE, become readily activated in the presence of Mg^{2+} ions and ATP. No nuclease contamination was found in any of the purified protein preparations, as evident from identical DNA profile of control and treated lanes (Fig. 3.9). The nuclease free protein preparations were used for EMSA, topoisomerase assays and strand exchange assays.

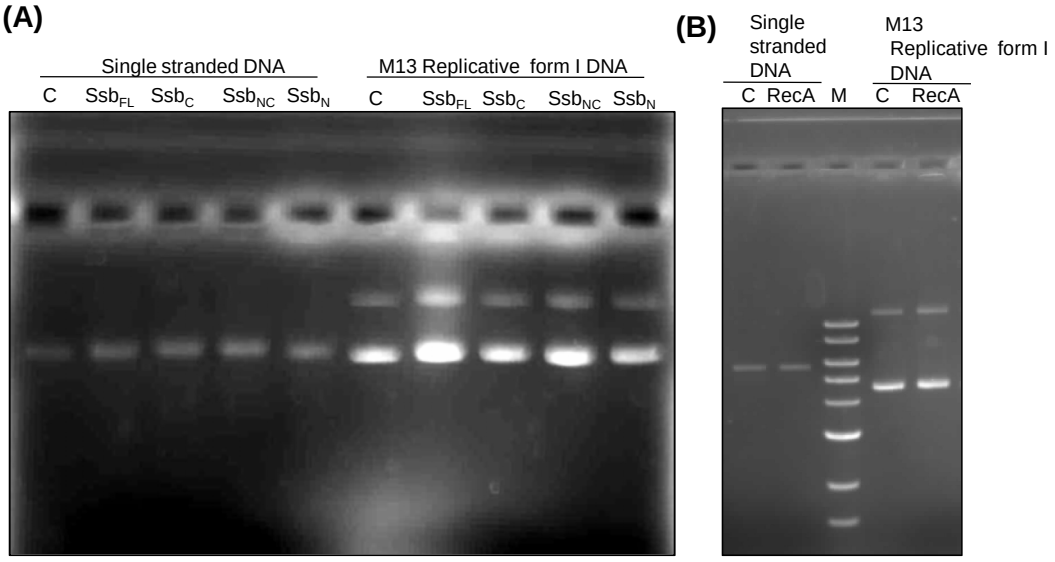


Fig. 3.9. Nuclease activity assay of all Ssb variants and RecA. (A) Standard nuclease assay was carried out with specified variants of Ssb (2 µM) with M13 single stranded and M13 replicative form I DNA (10 µM) as described above. Lane C is control lane (B) Standard nuclease assay carried out with Deinococcal RecA protein and M13 single stranded and M13 replicative form I DNA. Lane C is control lane. The reaction mix was incubated at 37°C for 1h and then electrophoretically resolved on 0.8% Agarose gel. The gel was stained by ethidium bromide after electrophoresis and visualized by UV illumination.

3.1.9. Determination of oligomeric status of Ssb_{FL}, Ssb_{C}, Ssb_{N} or Ssb_{NC}.

Oligomeric status determination was carried out in 2 different ways, i.e. by glutaraldehyde cross-linking followed by 14% SDS-PAGE and size exclusion chromatography using Superdex-75.

i) Oligomeric status of Deinococcal Ssb variants and Ssb_{FL} were assessed by glutaraldehyde cross linking. As shown in Figure 3.10, Ssb_{FL} (monomer 35 kDa with His-tag) was found to predominantly exist as a 70 kDa dimer.

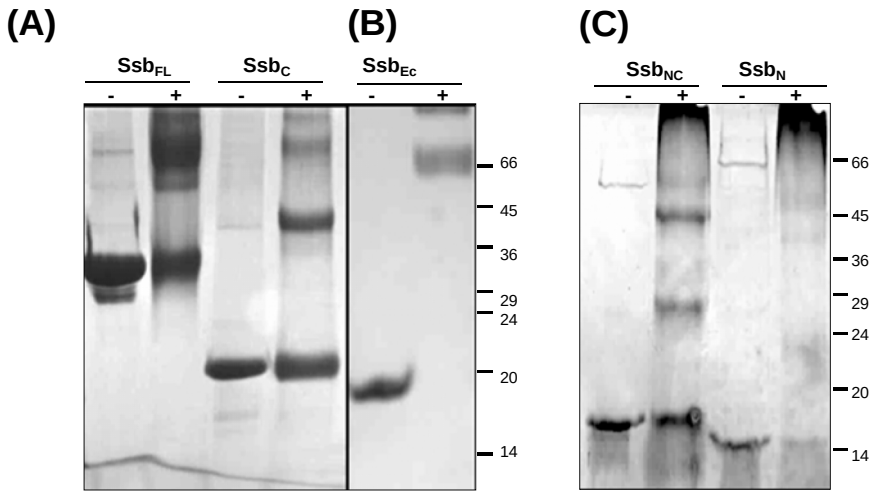


Fig. 3.10. Oligomeric status determination by glutaraldehyde cross-linking. About 25 µg each of Ssb_{FL}, Ssb_{C}, Ssb_{NC}, Ssb_{N} or *E. coli* Ssb proteins was individually treated with glutaraldehye (final concentration 0.125%) at 37°C for 5 mins and reaction terminated by adding 1 M Tris-HCl pH 8.0. Untreated (-) and glutaraldehyde treated (+) proteins ~ 5µg for (A) Ssb_{FL} and Ssb_{C} or (B) *E. coli* Ssb or ~ 2 µg of (C) Ssb_{NC} and Ssb_{N} were electrophoretically resolved by 14% SDS PAGE and stained with CBB. Molecular mass of co-electrophoresed SDS protein markers is depicted on the right hand side of the figures.

The N-terminal truncated Ssb_{C} protein (monomer 21.6 kDa with His-tag) was also found as a dimer (43.2 kDa), and a tetrameric form was also evident. In contrast, the *E. coli* Ssb (Ssb_{EC}, monomer 18 kDa) was found to be a tetramer (72 kDa) after cross-linking (Fig. 3.10). Ssb_{NC} and Ssb_{N} proteins displayed polydispersivity after cross-linking. Ssb_{NC} (monomer 14.7 kDa with additional 10 amino acids) formed dimer (29.4 kDa), trimer (44.1 kDa), tetramer (58.8 kDa) and other complex multimers. Ssb_{N} (monomer 13 kDa with additional 3 amino acids) similarly occurred in diverse multimeric forms. The dimeric (26kDa), trimeric (39kDa) and tetrameric (52 kDa) forms being easily discernible (Fig 3.10 C). Differences between the observed and theoretical molecular weights of Ssb_{NC} noted in this study may be attributable to the presence of two hydrophobic

amino acid patches (Y9, I11 & V78, 79, E80, 107, L109, T111, P113, E112) identified by using pymol software analysis (https://www.pymol.org/). Although, Ssb_C shares more similarity to *E. coli* Ssb at the amino acid level, it prominently occurred as a dimer as against tetrameric *E. coli* Ssb. Also, presence of connector appeared to control the multimerization property of Ssb_N.

ii) Oligomeric forms of Deinococcal Ssb variants were separated by gel filtration chromatography. Ssb_{FL} (monomer with His-tag - 35 kDa) was primarily found to exist as a dimer of 60.8 kDa (Figure 3.11) and Ssb_C (monomer with His-tag - 21.6 kDa) as a dimer of 46.2 kDa. Ssb_{NC} and Ssb_N proteins displayed polydispersivity. Ssb_{NC} (monomer 14.7 kDa with additional 10 amino acids) formed a dimer (22 kDa) as well as complex multimers of higher molecular mass, which eluted in the void volume of the column. Ssb_N (monomer 13 kDa with additional 3 amino acids) also existed in diverse oligomeric forms of which the dimeric form (23 kDa) and complex multimeric forms of high molecular mass were clearly visible as discrete peaks. Differences between the observed and theoretical molecular weights of Ssb_{NC} noted may be attributable to the presence of hydrophobic amino acid patches. The oligomerization pattern of Ssb variants clearly demonstrated that the multimerization property resided with the N-terminal domain of Ssb, and may help in stabilization of dimeric state of Ssb_{FL}. The difference in FPLC and glutaraldehyde data may be attributed to the resolving difference of the two techniques as both use all together different principle. Higher multimeric forms may form weakly and therefore not visualized by FPLC in Fig.3.11 but may be stabilized by glutaraldehyde crosslinking and visualized in Fig. 3.10

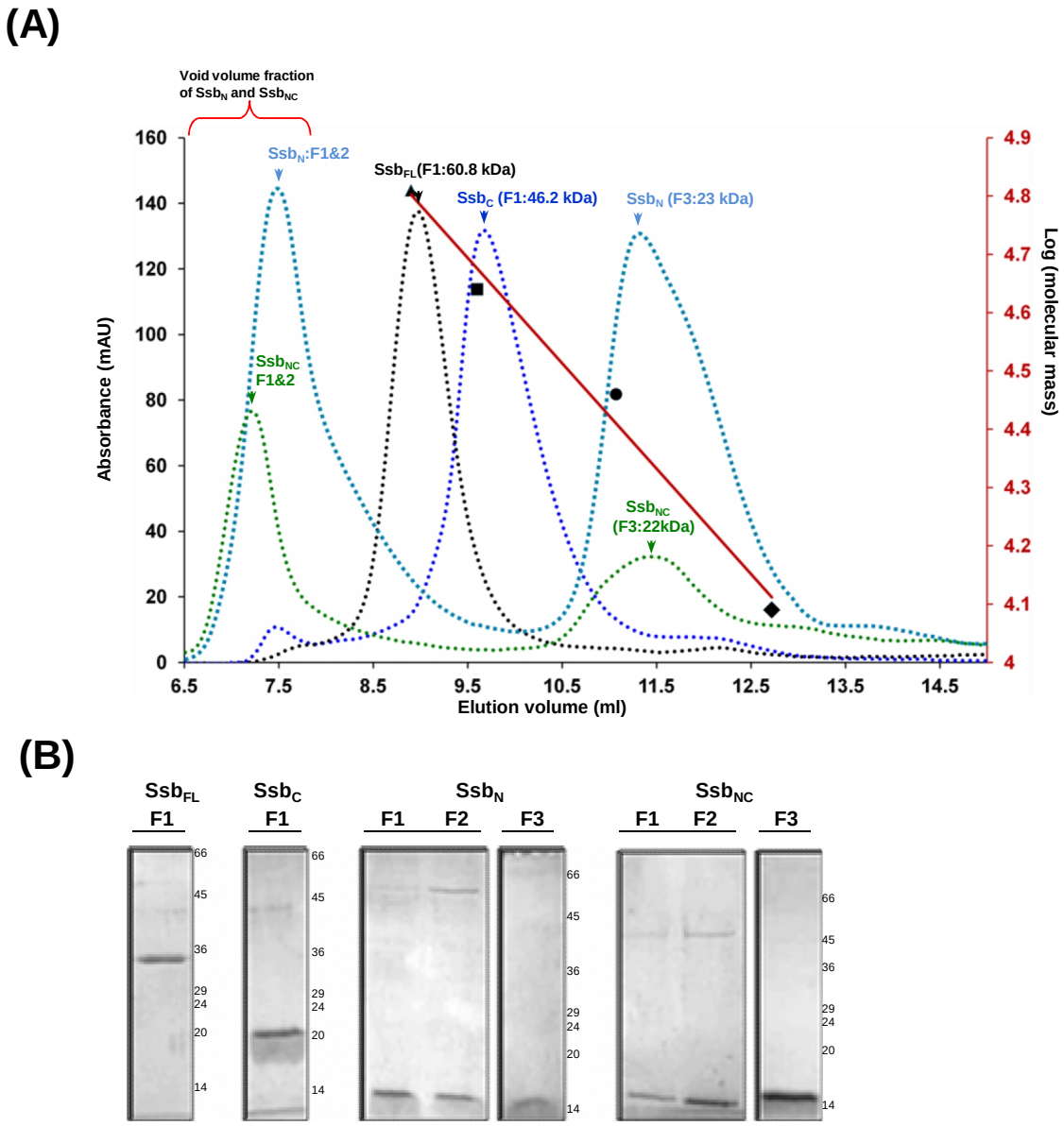


Fig. 3.11. Oligomeric status of Ssb variants as determined by gel exclusion chromatography. (A) Elution profile of all the Ssb variants is shown. Standard curve was drawn based upon the elution profile of the following protein standards: (▲, Bovine Serum Albumin: 66 kDa; ■, Ovalbumin: 44 kDa; ●, Carbonic anhydrase: 29 kDa; and ♦, Cytocrome C: 12.4 kDa). The continuous red line indicates linear fit of standard curve. The calculated molecular mass and fraction number of all Ssb variants are shown. Molecular mass of void volume fractions (F1 and F2) of Ssb_{NC} and Ssb_N could not be determined. (B) Purified fractions of Ssb variants shown in (A) were further resolved by 14% SDS PAGE. The fraction number of all Ssb variants is shown.

3.1 Biochemical Characterization of various Ssb variants

3.2.1 Binding of ssDNA to Ssb variants by EMSA (Electrophoretic Mobility Shift Assay).

The ssDNA binding activity of Deinococcal Ssb variants was evaluated by Electrophoretic Mobility Shift Assays (EMSA), using oligo dT50 and a range of protein concentrations (Figure 3.12). Among the various Ssb proteins, Ssb_{FL} displayed highest ssDNA binding activity [Fig. 3.12A (i)] followed by Ssb_C [Fig 3.12B (ii)]. The apparent equilibrium dissociation constant (K_D) was calculated to be 0.76±0.04 µM for Ssb_C (Hill coefficient: 4.25±0.48) as compared to 0.30±0.01 µM for Ssb_{FL} (Hill coefficient: 5.63±1.06), indicating that the ssDNA binding affinity of Ssb_C was lower than Ssb_{FL}. The Hill coefficients obtained were in agreement with those obtained earlier for other DNA binding proteins, including human Ssb protein [171, 172]. In comparison, Ssb_N and Ssb_{NC} bound ssDNA weakly and displayed different patterns of DNA-protein complexes [compare Fig. 3.12(C) with 3.12(D)]. Ssb_{NC} displayed a smear-like pattern and a distinct decrease in target DNA with increasing protein concentration [Fig. 3.12(D)], whereas Ssb_N showed discrete bands of DNA-protein complex but with no significant decrease in target DNA, commensurate with protein concentration. Cooperative ssDNA binding by Ssb_{FL} has been demonstrated earlier using long ssDNA molecules of M13 bacteriophage [17]. Among all the Ssb variants, Ssb_{FL} had highest affinity for ssDNA, followed by Ssb_C while the N-terminal region had the least. The presence of connector region appeared to improve ssDNA binding of the N-terminus.

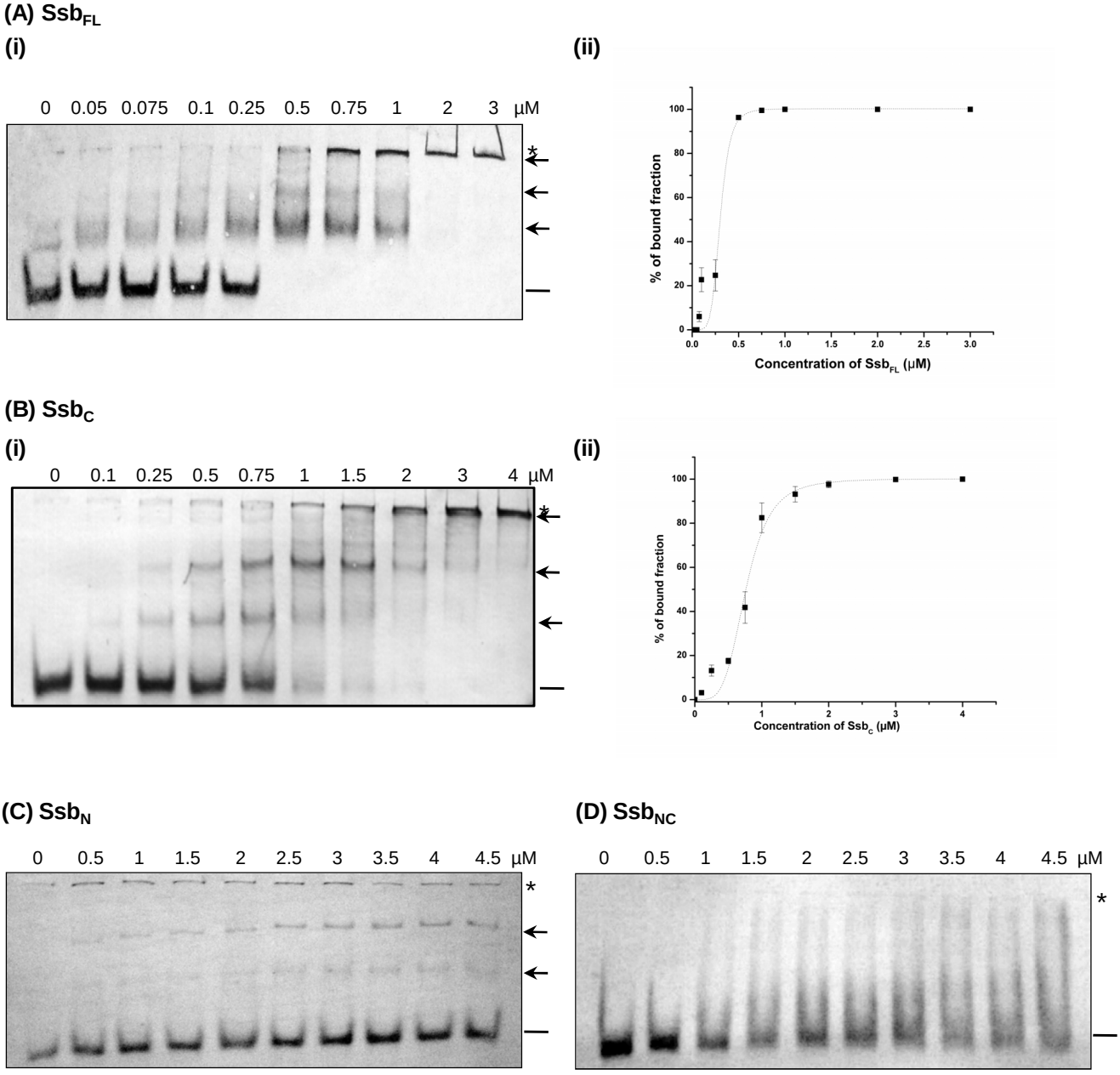


Fig. 3.12. Single-stranded DNA binding activity of Deinococcal Ssb variants. The indicated concentrations of Ssb protein variants were incubated with oligo dT50 (0.125 µM) for 20 min at 27°C and resolved by 12% native PAGE. The amount of protein-DNA complexes was quantified for Ssb_{FL} and Ssb_C using Gel Quant software. The representative graphs for Ssb_{FL} [panel A (ii)] and Ssb_C [panel B (ii)] are shown. The data points were fitted into Hill's equation (dotted line) to determine the K_D values. The error bars represent standard deviation of three independent experiments. DNA substrate and DNA:Ssb-protein complexes are shown by "—" and "←", respectively, while wells of the gels are marked by asterisk. (A) Ssb_{FL} (B) Ssb_C (C) Ssb_N (D) Ssb_{NC}

3.2.2. In vitro functional interaction of Ssb_N/Ssb_{NC} with Ssb_C

To test direct interaction between various Deinococcal Ssb variants, the indicated concentrations of Ssb_{NC} or Ssb_N (1-5 µM) were incubated with a fixed concentration of Ssb_C (0.125 or 0.25 µM), followed by addition of oligo dT50 (0.125 µM) (Fig. 3.13). At a limiting concentration of only Ssb_C (0.125 µM or 0.25 µM), no measurable ssDNA binding activity was observed (Fig. 3.13 A, B). However, in the presence of Ssb_C (0.125 µM), the amount of DNA-protein complexes increased with increasing concentration of Ssb_{NC} or Ssb_N (1–5 µM) (Fig. 3.13 A, B). Also, when Ssb_C concentration was increased to 0.25 µM, the amount of DNA-protein complex increased further (Fig. 3.13C, D). Irrespective of the Ssb_C concentration used, interaction of Ssb_N with Ssb_C resulted in a single ssDNA-protein complex (Figure 3.13 A, C), whereas interaction of Ssb_{NC} with Ssb_C resulted in 2 distinct protein-ssDNA complexes (Figure 3.13 B, D). The Ssb_N/Ssb_{NC} with Ssb_C complexes showed different binding pattern, which could be attributable to the connector region via additional protein-protein interactions. At a fixed Ssb_C concentration of 0.25 µM and 5 µM of Ssb_{NC}, the formation of a well-defined Ssb_{NC}/Ssb_C complex was observed, indicating that saturation had been achieved (Figure 3.13 D). Polydispersive forms of Ssb_N and Ssb_{NC} were separated by gel chromatography. Dimeric and multimeric fractions were obtained for both proteins, which displayed comparable ssDNA binding capacity, either alone or in association with $Ssb_{C,}$ similar to the un separated pool of proteins (Figure 3.13 E, F).

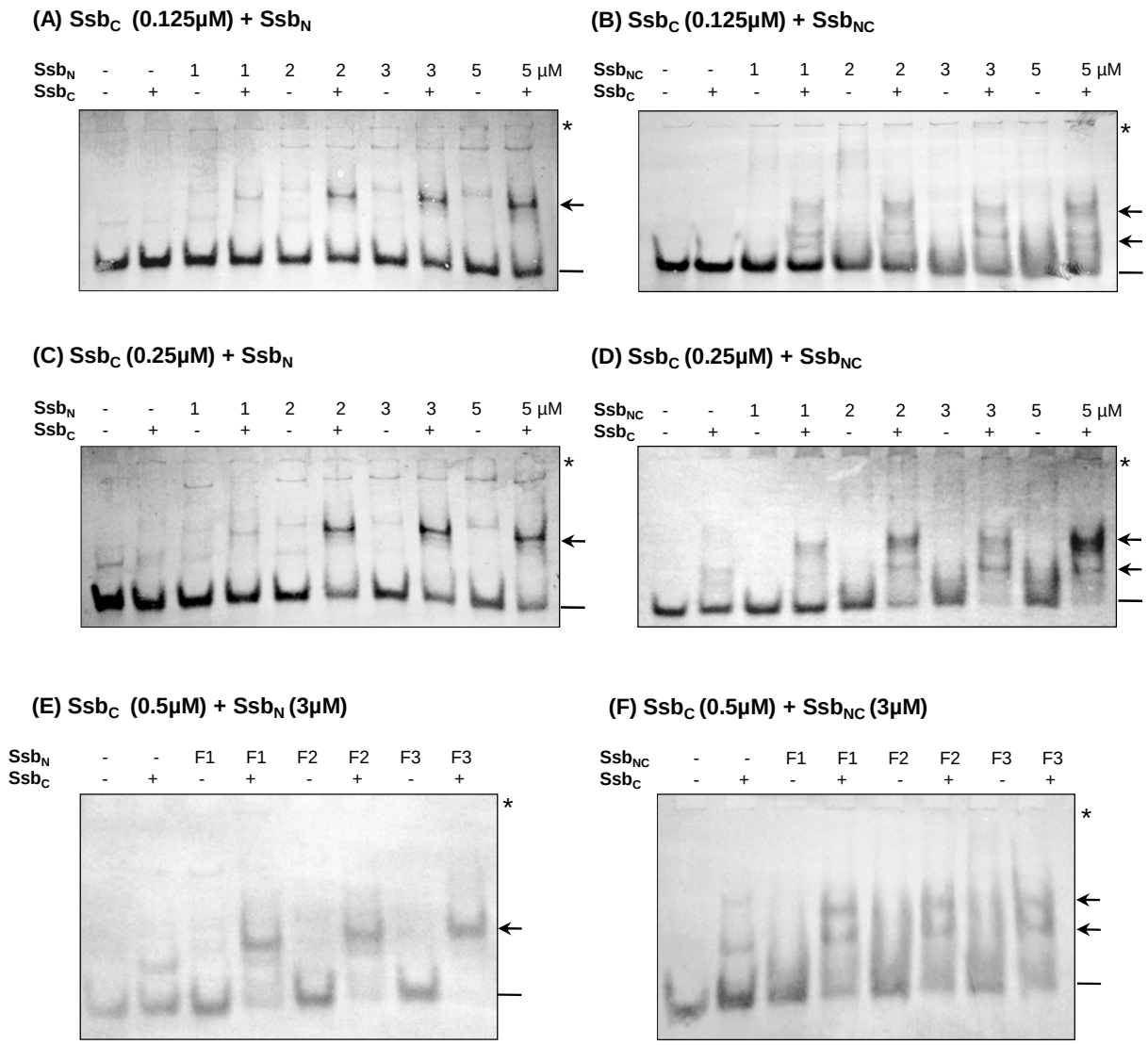


Fig. 3.13. *In vitro* functional interactions of Deinococcal $Ssb_{N/NC}$ with $Ssb_{C.}$ (A-B) EMSA was carried out wherein indicated concentration of Ssb_N (A) or Ssb_{NC} (B) were mixed with fixed concentration of Ssb_C (0.125 µM) and then incubated with 0.125 µM oligo dT50. The presence or absence of Ssb_C protein is indicated by "+" or "-" sign above each lane. (C-D) EMSA was carried out as detailed in (A-B), except that concentration of Ssb_C was fixed at 0.25 µM. (E-F). Combination EMSA was carried out using Ssb_C (0.5 µM) and various fractions of Ssb_N [(3 µM), Panel E] or Ssb_{NC} [(3 µM), Panel F] that were separated by Superdex-75 gel exclusion chromatography [F1&F2 are multimeric fraction and F3 is dimeric fraction of protein (Fig. 3.11)]. DNA substrate and Ssb-protein complex are shown by "—" and "←", respectively, while wells of the gels are marked by asterisk.

3.2.3. Assessment of the effect of $Ssb_{NC/N}$ on *E. coli* Ssb.

Deinococcal Ssb_C resembled *E. coli* Ssb. Therefore, the ability of Ssb_{NC} or Ssb_N, if any, to complement the ssDNA binding activity of *E. coli* Ssb at a limiting concentration was evaluated (Figure 3.14). Ssb_{NC} or Ssb_N (3-5 µM) did not functionally complement ssDNA binding activity of *E. coli* Ssb as no improvement in the yield of DNA-protein complexes was seen. These data suggest that the $Ssb_{NC/N}$ specifically interact with cognate Deinococcal Ssb_C only.

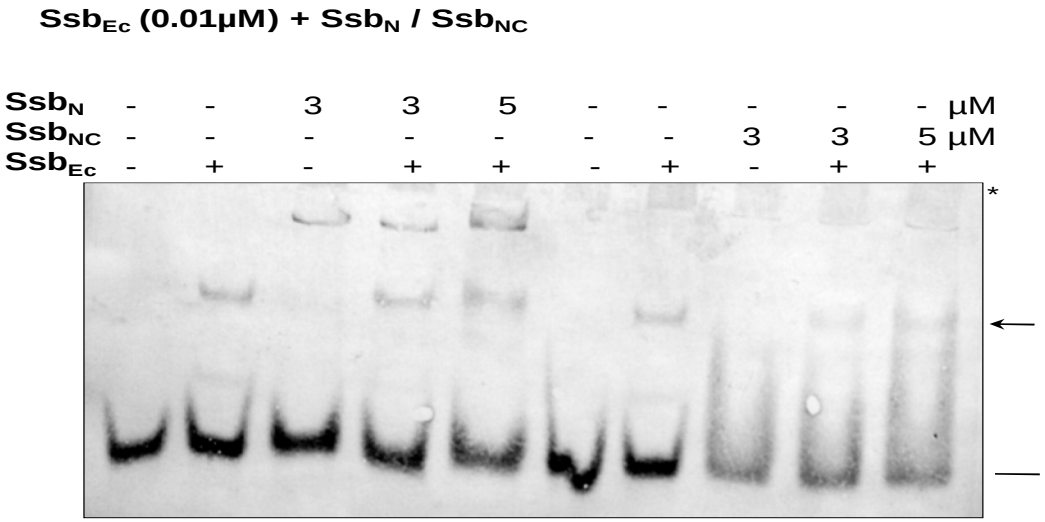


Fig. 3.14. *In vitro* functional interactions of Deinococcal $Ssb_{N/NC}$ with *E. coli* Ssb. The indicated concentrations of Ssb variants in combination with *E. coli* Ssb protein (0.01 µM) were incubated with oligo dT50 (0.125 µM) for 20 min at 27°C and resolved by 12% native PAGE. DNA substrate and DNA:Ssb-protein complex are shown by "—" and "←", respectively while wells of the gels are marked by asterisk.

3.2.4. Physical interaction of Ssb_{NC}/Ssb_N with Ssb_C, as assessed by affinity chromatography.

Direct physical interaction of Ssb_{NC} or Ssb_N with Ssb_C was ascertained, *in vitro*, using affinity chromatography as a test. Ssb_C was first bound to Ni-NTA agarose column through its His-tag and purified Ssb_{NC} or Ssb_N (both without any tag) were then individually passed through the column. After extensive washing, the column was eluted with 250 mM immidazole. Analysis of fractions indicated that Ssb_{NC} or Ssb_N co-eluted with Ssb_C, thereby confirming physical interaction of N-terminal domain of Deinococcal Ssb with its C-terminal domain (Figure 3.15). In reverse binding method, Ssb_N with chitin binding domain was bound to chitin beads and *E. coli* crude extract containing over expressed Ssb_C was equilibrated in the column. *E. coli* crude extract containing overexpressed Ssb_C was purposefully used in this experiment to provide more competition to Ssb_N in binding Ssb_C. The column was subsequently washed and tag of Ssb_N was removed by overnight treatment of DTT. The eluted fractions contained both Ssb_N and Ssb_C (Figure 3.15B). The aforesaid two experiments provide additional evidence that N-terminal portion physically interacts with C-terminal portion of Deinococcal Ssb. The observation that Ssb_{NC} or Ssb_N interact both physically and functionally with Ssb_C suggest that correct folding of the whole complex may lead to higher affinity for ssDNA as compared to Ssb_C alone.

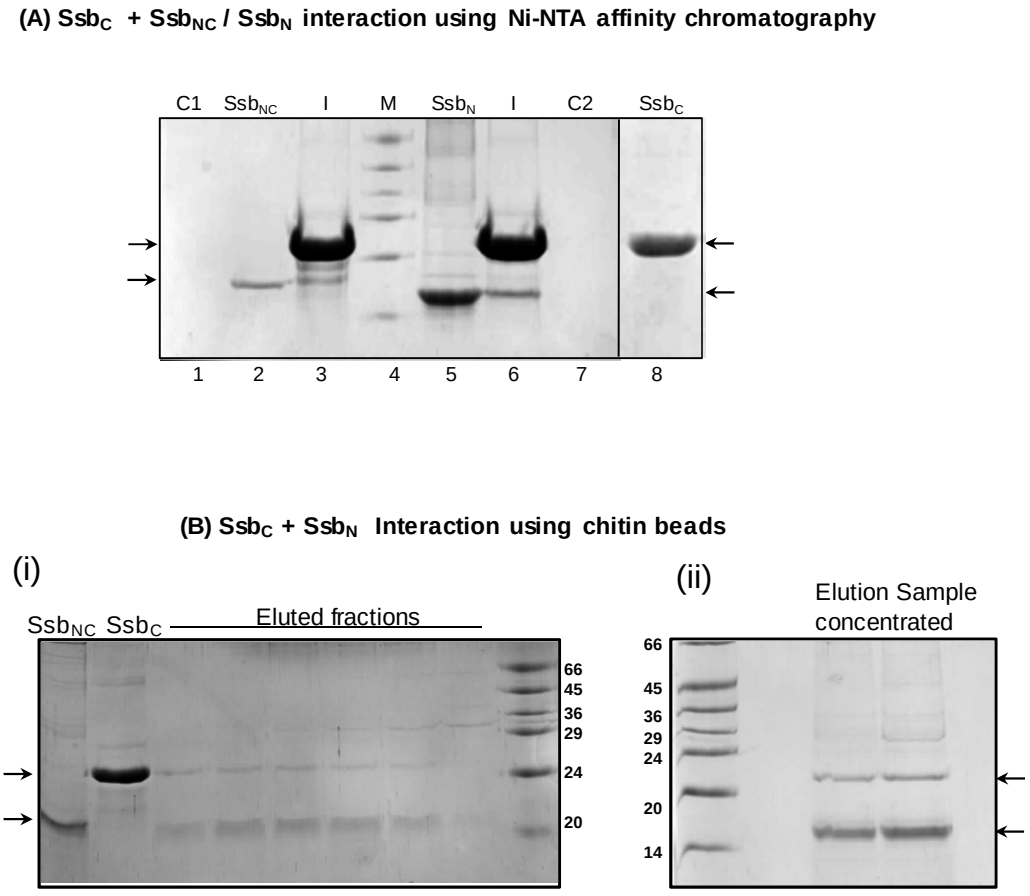


Fig. 3.15. *In vitro* interaction of N-terminal domain with C-terminal domain of Deinococcal Ssb, as assessed by affinity chromatography. (A) Ssb_C (bait) was bound to Ni-NTA by its His-tag and the column was equilibrated with purified Ssb_N or Ssb_{NC} (prey). Subsequently the columns were washed and eluted by increasing immidazole concentration (250 mM). The column elutants (lane 3, Ssb_{NC} and $Ssb_{C,}$ and lane 6, Ssb_N and Ssb_C) were resolved by 14% SDS PAGE. Proteins in Lanes 2, 5 and 8 contained Ssb_{NC}, Ssb_N and Ssb_C, respectively for easy assessment of prey and bait proteins. Lane 4 contained protein molecular mass marker (SDS7, Sigma, India). C1 and C2 (Lane 1 and 7) contained eluted fraction of Ssb_{NC} and Ssb_N treated similarly but in the absence of Ssb_C (bait) protein. (B) In reverse binding analysis, Ssb_N with chitin binding domain (bait) was bound to chitin column and equilibrated with *E. coli* extract containing over expressed Ssb_C (prey). The column was washed and eluted by overnight DTT treatment. Lanes Ssb_N and Ssb_C contained purified proteins as protein markers, lanes 1-3 in (i) are various intermediate eluted fractions. In (ii) the eluted fractions were concentrated and resolved by 14% SDS-PAGE. Protein marker (SDS-7) was used as molecular weight marker.

3.2.5. Competition EMSA of Ssb_{FL} with other Ssb variants.

The effect of other Ssb variants on the binding of Ssb_{FL} to ssDNA was assessed by EMSA in a competition assay, wherein the amount of Ssb_{FL} was kept constant at 0.5 µM and titrated against increasing concentration of Ssb_C (Fig. 3.16A), Ssb_N (Fig. 3.16B) and Ssb_{NC} (Fig. 3.16C). No Ssb variant was found to influence Ssb_{FL} binding to ssDNA.

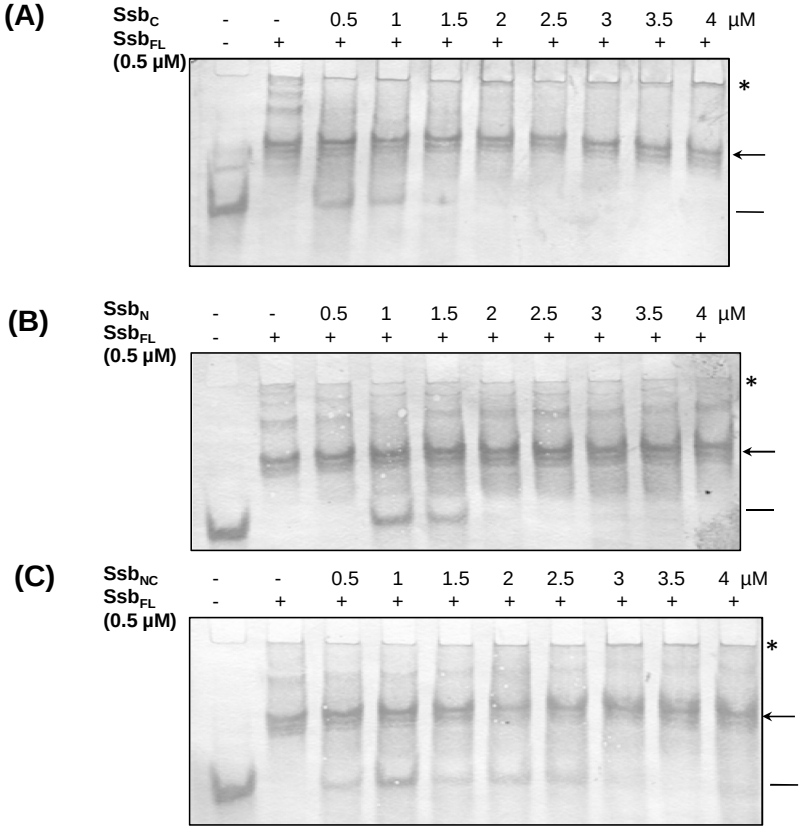


Fig. 3.16. Competition EMSA of Ssb_{FL} with other Ssb variants. Ssb_{FL} protein at 0.5 µM was mixed with indicated concentrations of other Ssb variants and then incubated with oligo dT50 (0.125 µM) for 20 min at 27°C and resolved by 12% native PAGE. DNA substrate and DNA:Ssb-protein complexes are shown by "—" and "←", respectively, while wells of the gels are marked by an asterisk. (A) Ssb_{FL}+ Ssb_C (B) Ssb_{FL}+Ssb_N (C) Ssb_{FL}+Ssb_{NC}.

EMSA studies clearly indicated that the DNA binding ability of Deinococcal Ssb resides largely in the C-terminal domain, while multimerization property lies with N-terminal domain. *In vitro* combination assays of the two domains showed that N-terminal region also contributes significantly in forming functional protein complex with C-terminal domain, which has higher affinity for ssDNA in comparison to both individual ($Ssb_{N/NC}$ or Ssb_{C}) domains.

In order to test the ability of all Ssb variants and their combination in executing more complex reactions in comparison to mere binding to DNA as shown by EMSA, effect of Ssb variants on topoisomerase activity was analyzed using *E. coli* topoisomerase I. Ssb modulates the activity of many DNA binding proteins including topoisomerase I and RecA. Ssb enhances topoisomerase I activity by stabilizing localized DNA melting at the site of topoisomerase interaction on negatively supercolied dsDNA (ds DNA) substrate. This localized DNA melting acts as a perfect spot and seeding point for topoisomerase I action [154]. Since differential binding of all the Ssb variants and their combination was observed for oligo dT50, their effect was also studied on topoisomerase activity.

3.3. Topoisomerase I assays with various Ssb variants.

3.3.1. Standardization of concentration of Topoisomerase I to be used.

Topoisomerase I relaxes dsDNA to a relaxed molecule in an ATP-independent reaction at 37°C. The reaction is aided by Ssb protein. The concentration of topoisomerase I to be used in the reaction should be such that only limited relaxation of DNA occurs and can be enhanced further by Ssb. The assay conditions were therefore standardized. About 0.1 units

of *E. coli* topoisomerase I did induce limited relaxation of DNA alone (Fig. 3.17) and effects of all Ssb variants or their combination could be assessed easily.

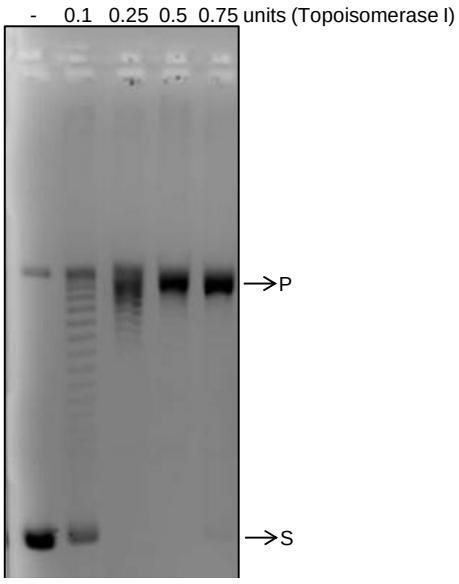


Fig. 3.17. Standardization of concentration of *E. coli* topoisomerase I to be used. *E. coli* topoisomerase I at concentrations indicated above the lanes was added to 0.5 µg of M13 dsDNA. The reaction was carried out for 15 mins and then terminated by addition of 10 mM EDTA followed by heat inactivation. The reaction mix was electrophoretically resolved overnight by 0.8% agarose at 23V, stained with ethidium bromide and visualized under UV. Substrate and product are marked as "S" and "P" respectively.

3.3.2 Topoisomerase assay with Ssb_{FL}.

The effect of Deinococcal Ssb_{FL} on *E. coli* topoisomerase I activity was checked using dsM13DNA. Topoisomerase I formed a single major band of relaxed DNA in the presence of Ssb_{FL} [Fig. 3.18 (lanes 3-14)]. No intermediate topoisomeres were seen even at lowest concentration (0.25 µM) of Ssb_{FL}. Topoisomerase I, thus showed exclusively processive behavior of forming single relaxed molecule in the presence of Ssb_{FL}.

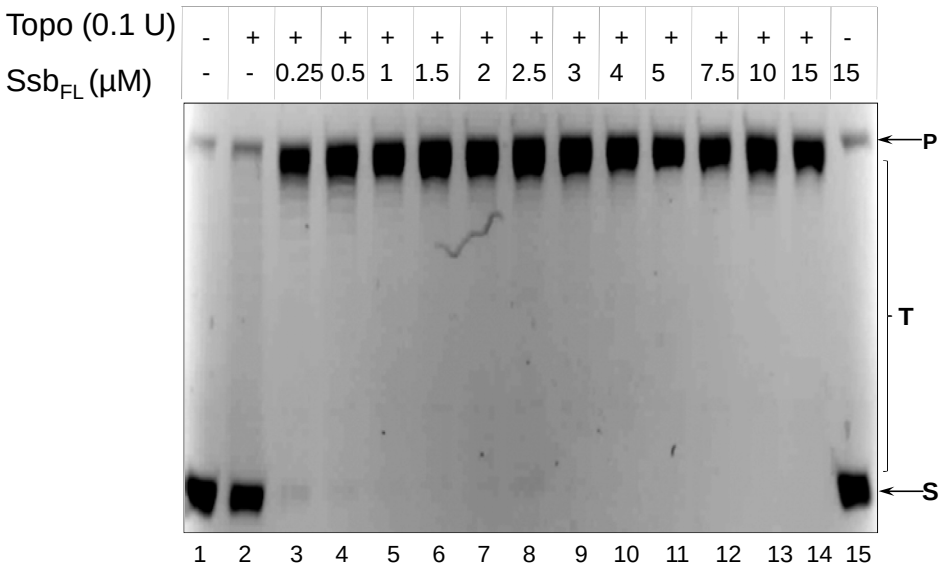


Fig. 3.18. Modulation of *E. coli* topoisomerase activity by Deinococcal Ssb_{FL}. Supercoiled M13mp18RFI DNA was incubated with (+) or without (-) *E. coli* topoisomerase I (0.1 unit) in the absence (-) or presence (+) of the indicated concentration of Deinococcal Ssb_{FL}. The reaction mixture was processed as described in Fig 3.17. The concentrations of Ssb_{FL} are indicated at the top of each lane. Lane 15 contained the substrate control. Substrate and product are marked as "S" and "P" respectively, no intermediate topoisomers "T" were seen.

3.3.3. Topoisomerase assay with Ssb_C, Ssb_{NC}, Ssb_N or their combination.

The effect of Deinococcal Ssb_C, Ssb_{NC}, Ssb_N or their combination on *E. coli* topoisomerase I activity was checked using dsM13DNA. In the presence of Ssb_C, Ssb_{NC} or Ssb_N discernible generation of intermediate DNA topoisomers was observed. In contrast to Ssb_{FL},

Topoisomerase I showed distributive behavior with all truncated Ssb variants (Ssb_C, Ssb_N, and Ssb_{NC}) or their combination. In the presence of N-terminal domains (Ssb_{NC} and Ssb_N) topoisomerase I generated intermediate topoisomers at lower concentration of Ssb_{NC} and Ssb_N (Fig.3.19 A,B lane 5, 8, 11, 14), but showed inhibition at higher concentrations of these proteins (Fig.3.19 A,B lane 19, 22, 25, 28). In the presence of Ssb_C, topoisomerase I resulted in a higher yield of relaxed DNA at higher concentration than at lower concentration of Ssb_C (Figure 3.19 A, B lane 18, 21, 24, 27). The data indicate that both the OB folds of Deinococcal Ssb were independently capable of melting ssDNA locally, thereby enhancing topoisomerase I activity. However, Ssb_{NC}/Ssb_N appeared to inhibit topoisomerase I activity at higher concentrations and their addition decreased the positive effect of Ssb_C on topoisomerase I activity. The modulation in topoisomerase activity may be due to stabilization of single-stranded DNA formed at the site of topoisomerase action. The variation seen in the pattern of DNA topoisomers generated by $Ssb_{NC/N}$ or Ssb_C may be attributable to their indiviual affinity for single-stranded DNA formed at topoisomerase action site as well as accessibility to such sites. Following gamma irradiation, accumulation of high concentration Ssb protein and its C-terminal processing is well documented in *Deinococcus radiodurans* [7, 163]. The data presented (Fig. 3.19) suggest that the individual N and C terminal Ssb domains can aid the activity of native topoisomerases during DNA damage repair in the recovery phase.

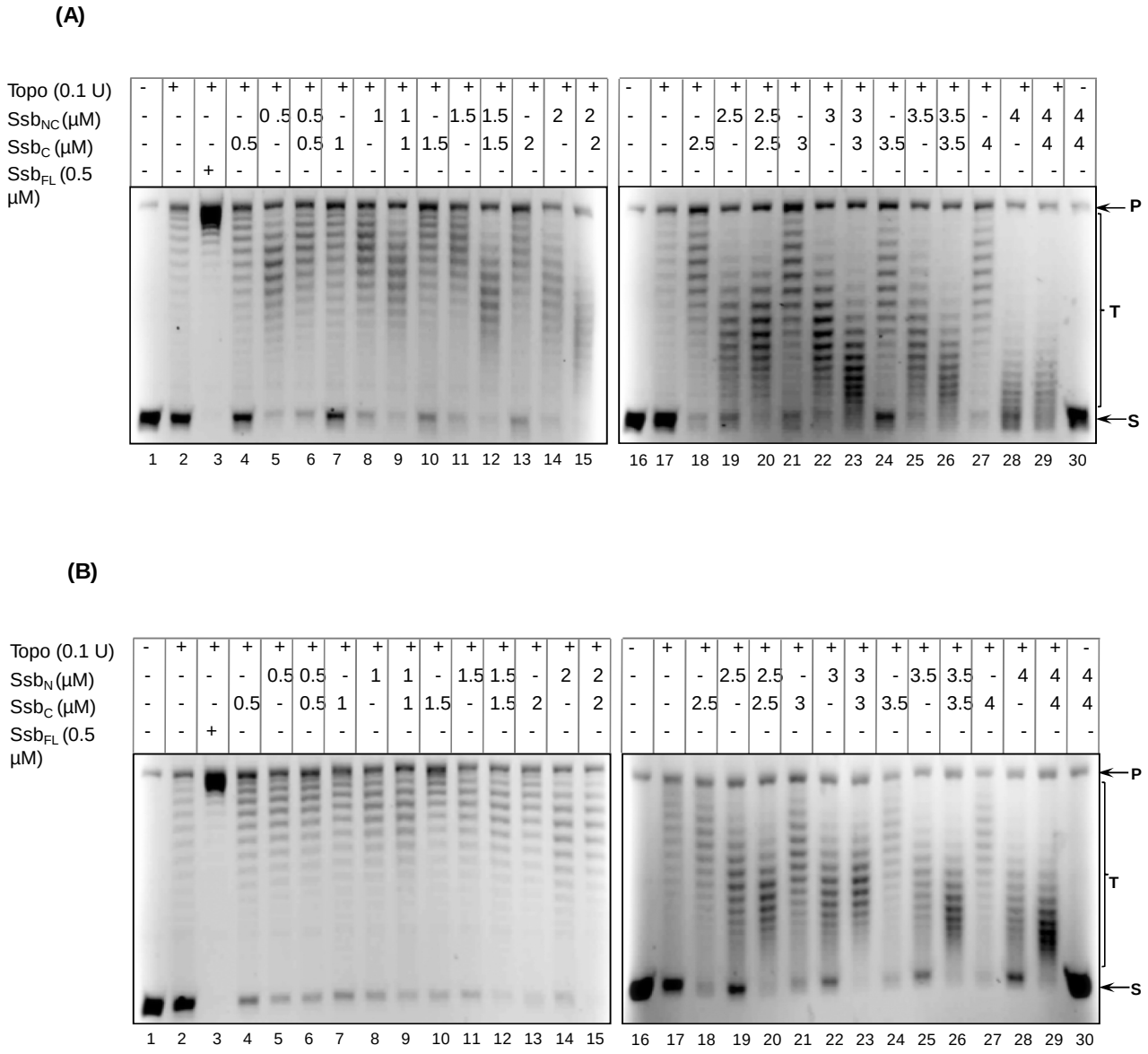


Fig. 3.19. Modulation of *E. coli* topoisomerase activity by Deinococcal Ssb variants. (A) Heterologous *E. coli* topoisomerase I (0.1 unit) was incubated with indicated concentrations of Deinococcal Ssb variants (Ssb_C and Ssb_{NC}), either alone or in combination with 500 ng of supercoiled M13mp18RFI form DNA for 15 mins at 37°C. The reaction mixture was processed as described in Fig 3.17. The concentrations of various Ssb variants used are indicated on the top of each lane. (B) Topoisomerase assay similar to described in (A), except that Ssb_N was used instead of Ssb_{NC}. The 30th lane in (A) and (B) contained substrate control while lane 3 contained Ssb_{FL} (0.5 μM) as a positive control. Substrate, product and intermediate topoisomers are marked as "S", "P" and "T" respectively.

3.3.4. Competition Topoisomerase assay with Ssb_{FL} and Ssb_C.

As Topoisomerase I showed processive behavior with Ssb_{FL} and distributive behavior with all other Ssb variants alone or in combination, the topoisomerase activity was also tested in the presence of combination of Ssb_{FL} with individual Ssb variants. Increasing concentration of Ssb_C did not affect the processive behavior of Topoisomerase I in the presence of fixed concentration of Ssb_{FL} (Fig 3.20).

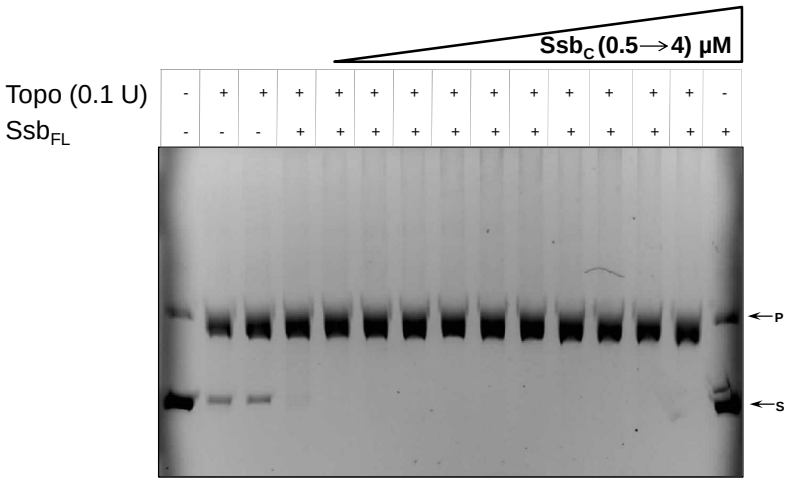


Fig. 3.20. Competition Topoisomerase assay involving Ssb_{FL} and Ssb_C. Heterologous *E. coli* topoisomerase I (0.1 unit) was incubated with 0.125 µM of Deinococcal Ssb_{FL} and increasing concentration of Ssb_C with 500ng of supercoiled M13mp18RFI form DNA for 15 mins at 37°C. The reaction was carried out and products resolved as described previously (Fig 3.17). The components added in each lane are indicated on the top of the lanes. Substrate and product are marked as "S" and "P" respectively.

3.3.5. Competition Topoisomerase assay with Ssb_{FL} and Ssb_{NC} or Ssb_N.

The processive behavior of Topoisomerase I observed with Ssb_{FL} was also not affected by increasing concentration of Ssb_N or Ssb_{NC} (Fig. 3.21). The competition data of Ssb_C, Ssb_N and Ssb_{NC} suggest that Ssb_{FL} has the highest affinity and strongest influence on the reaction site of topoisomerase action than any of the other Ssb variants.

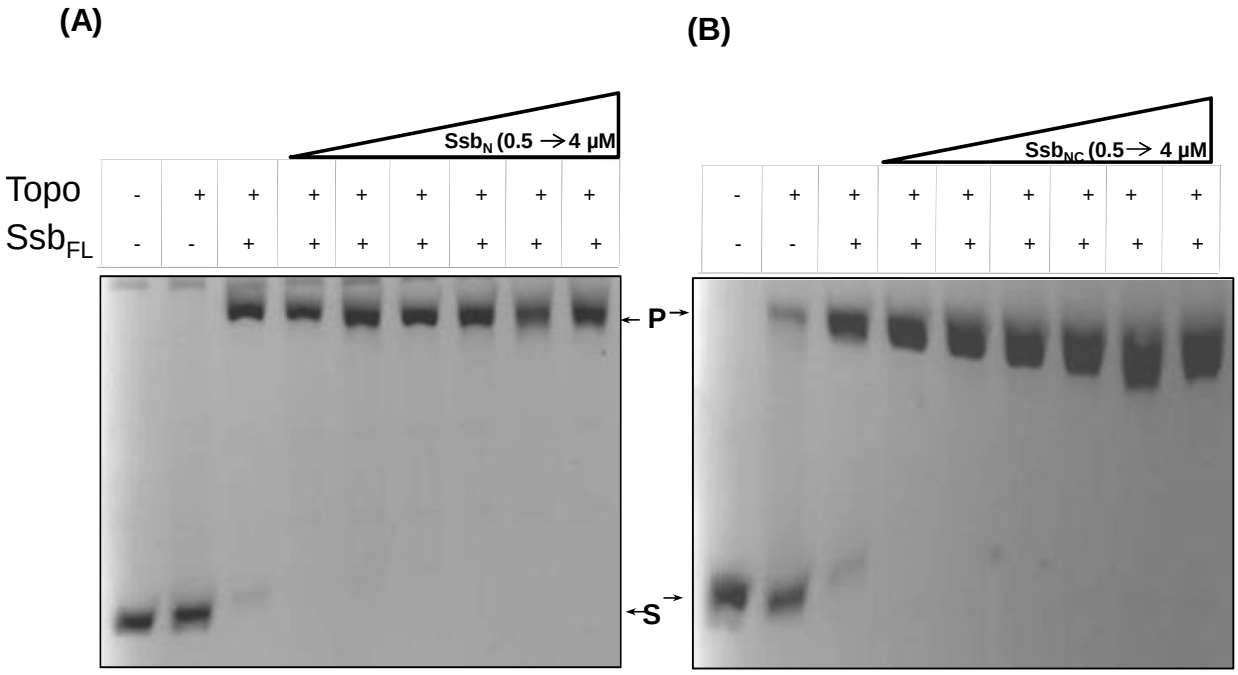


Fig. 3.21. Competition Topoisomerase assay involving Ssb_{FL} and Ssb_N or Ssb_{NC}. Heterologous *E. coli* topoisomerase I (0.1 unit) was incubated with 0.125 µM of Deinococcal Ssb_{FL} and increasing concentration of Ssb_N (A) or Ssb_{NC} (B) with 500ng of supercoiled M13mp18RFI form DNA for 15 mins at 37°C. The reaction was carried out and products resolved as described previously (Fig 3.17). The components added in each lane are indicated at the top of the lanes. Substrate and product are marked as "S" and "P" respectively.

All the Ssb variants and their combinations differentially modulated Topoisomerase I activity by stabilizing single stranded region at topoisomerase I site. More complex activity, shown by all other Ssb's, of removing secondary structure of ssDNA, to enhance RecA mediated strand exchange activity was examined next with all Deinococcal Ssb variants and their combination.

3.4. Strand Exchange Assays.

RecA protein is important for DNA recombination and repair. The activity of RecA was assessed by strand exchange assay. RecA executes strand exchange from linear double stranded DNA onto circular ssDNA (substrates) leading to generation of nicked circular (product) molecule. Single stranded DNA binding protein plays a very important role in strand exchange assays, wherein it removes the secondary structures of single stranded DNA substrate thus enhancing the RecA activity as well as preventing the reverse reaction by the displaced single strand from previous strand exchange [120]. Deinococcal RecA activity was carried out in two modes, namely the SS-DS and DS-SS modes [114]. In SS-DS mode, RecA was first incubated with ssDNA, followed by addition of Ssb and dsDNA was added last. In DS-SS mode, RecA was first incubated with dsDNA followed by addition of ssDNA and Ssb was added at the end.

3.4.1. Ssb_{FL} enhances Deinococcal as well as *E. coli* RecA activity.

i) Standardization of substrate ratio in strand exchange assay.

Strand exchange reaction requires ssDNA and linear dsDNA as substrate, which are converted to nicked circular molecule products upon RecA activity. The ratios of the two substrates affect the amount of product formed. The reactions were carried out with substrate ratios of 1:1 and 1:2 (ssDNA: linear dsDNA) in reaction buffer containing ATP and ATP regeneration system and Deinococcal RecA as well as Ssb_{FL}. It was found that the substrate ratio of 1:1 provided greater quantity of nicked circular molecules (Fig. 3.22). Therefore substrate ratio of 1:1 was subsequently used for all of the further studies.

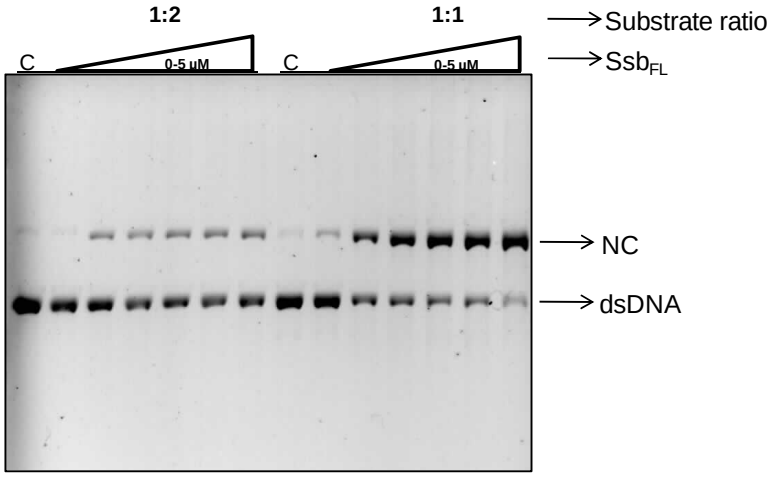


Fig. 3.22. DNA strand exchange assay. Assay was performed with Deinococcal RecA (3 µM) in the presence of Ssb_{FL} with substrate ratio of 1:2 and 1:1. The reaction was done for 1.5 h and then terminated by adding SDS, products were electrohoretically resolved overnight on 0.8% agarose gel at 23V and subsequently stained by ethidium bromide, to be able to visualized under UV. Lane "C", in all the gels contained RecA but do not contain any Ssb_{FL}. Substrate (dsDNA) and product, nicked circular DNA molecule (NC) are marked.

ii) Ssb_{FL} enhances nicked circular DNA formation from both Deinococcal RecA as well as *E. coli* RecA. The ability of Deinococcal Ssb_{FL} to stimulate DNA strand exchange activity of cognate Deinococcal RecA and non cognate *E. coli* RecA was assessed. The reaction with Deinococcal RecA was carried out in two modes, SS-DS and DS-SS, while with *E. coli* RecA it was carried out in SS-DS mode. Addition of Ssb_{FL} (up to 3 µM) increased the efficiency of RecA dependent strand exchange, as seen by the extent of formation of nicked circular DNA, both in the SS-DS mode as well as in the DS-SS mode. The results obtained with Deinococcal RecA as well as in *E. coli* RecA in SS-DS mode were similar (Figure 3.23).

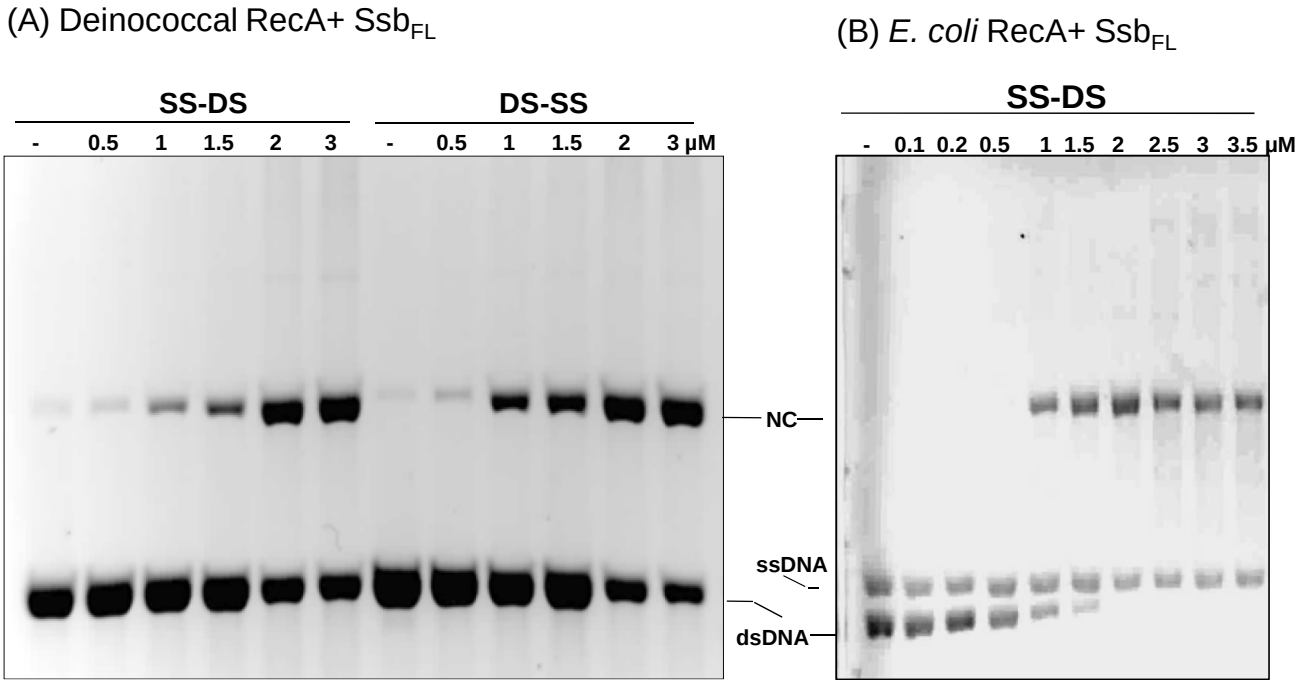


Fig. 3.23. DNA strand exchange assay with Ssb_{FL}. Assay was performed with (A) Deinococcal RecA or (B) *E. coli* RecA in the presence of increasing concentration of Ssb_{FL}. The reaction mix was processed as described in Fig 3.22. Lane '-', in the gels (A) and (B) contained RecA but no Ssb_{FL}. Substrate ssDNA and linear dsDNA are marked and product is marked as "NC" respectively.

3.4.2. Ssb_C does not enhance strand exchange by Deinococcal or *E. coli* RecA.

Strand exchange assay was also performed with Ssb_C. Ssb_C had no noticeable effect and did not enhance formation of nicked circular product molecules (Fig. 3.24).

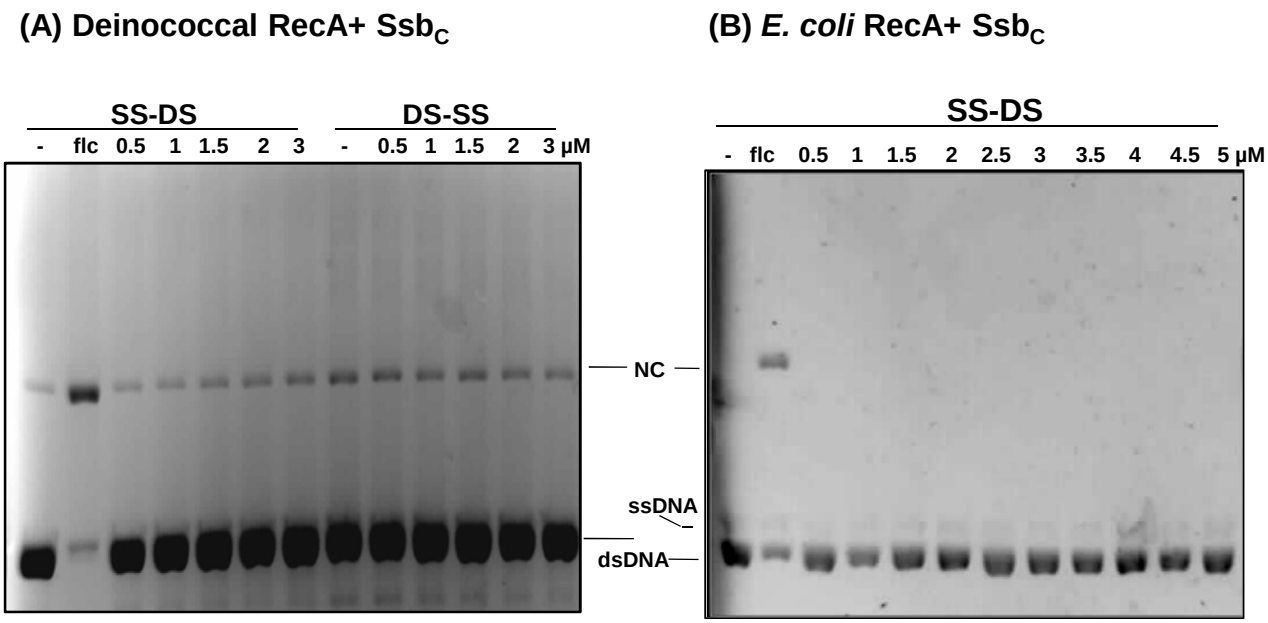


Fig. 3.24. DNA strand exchange assay with Ssb_C. Assay was performed with (A) Deinococcal RecA or (B) *E. coli* RecA in the presence of increasing concentration of Ssb_C. The reaction mix was processed as described in Fig 3.22 Lane '-', in gels (A) and (B) contains RecA but no Ssb_C. Substrate ssDNA and linear dsDNA are marked and product is marked as "NC" respectively.

3.4.3. Ssb_N and Ssb_{NC} do not enhance strand exchange by Deinococcal or *E. coli* RecA

Strand exchange assay was also performed with Ssb_N or Ssb_{NC} proteins. It was found that Ssb_N (Fig. 3.25) or Ssb_{NC} proteins (Fig. 3.26) neither enhanced formation of nicked circular product molecules with Deinococcal RecA, nor with *E. coli* RecA. Moreover at higher concentrations of Ssb_N or Ssb_{NC}, the RecA activity was inhibited.

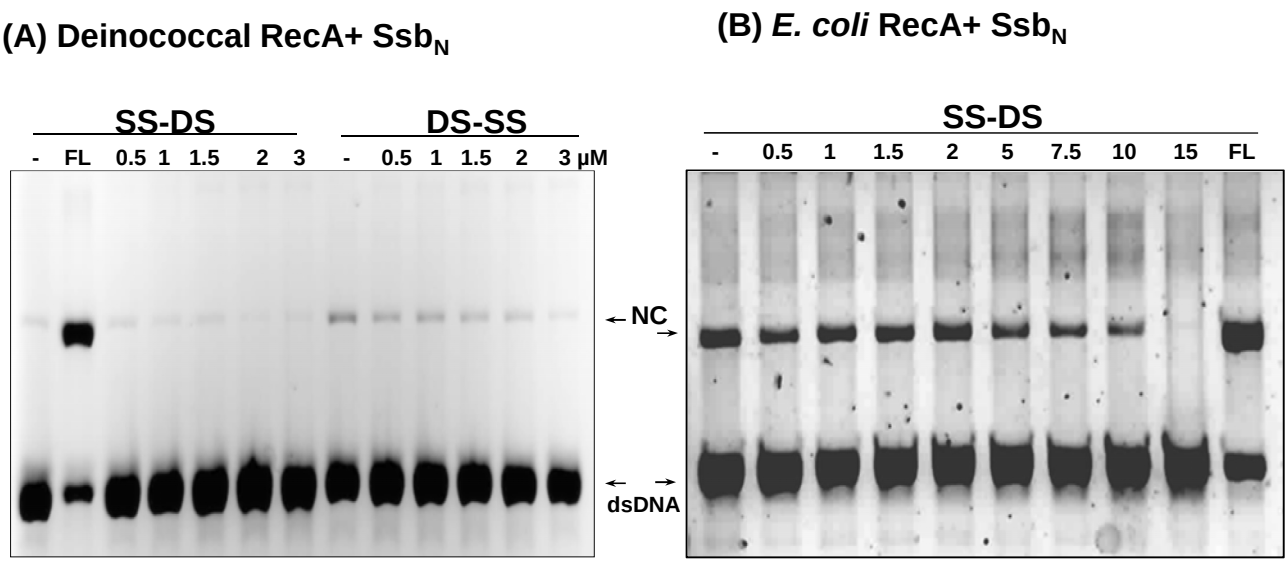


Fig. 3.25. DNA strand exchange assay with Ssb_N. Assay was performed with (A) Deinococcal RecA or (B) *E. coli* RecA in the presence of increasing concentration of Ssb_N. The reaction mix was processed as described in fig 3.22. Lane '-', in all the gels contains RecA but no Ssb_N. Substrate dsDNA is marked and product is marked as "NC" respectively. The substrates used for Deinococcal RecA were Ø174 ssDNA and linear Ø174 dsDNA, whereas for *E. coli* RecA the substrates used were M13ssDNA and linear M13dsDNA.

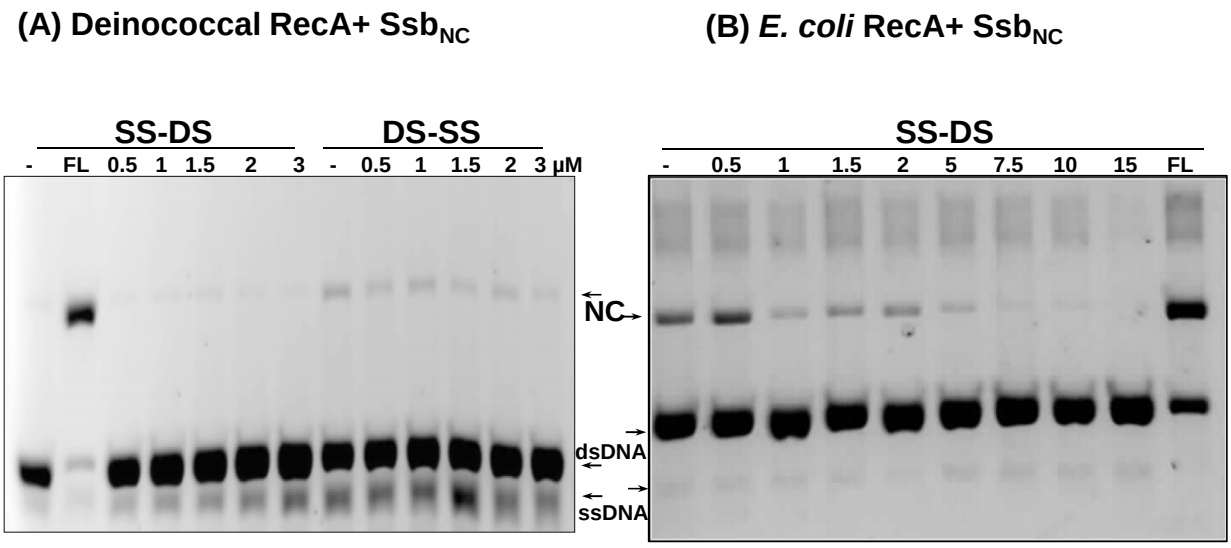


Fig. 3.26. DNA strand exchange assay with Ssb_{NC}. Assay was performed with (A) Deinococcal RecA or (B) *E. coli* RecA in the presence of increasing concentration of Ssb_{NC}. The reaction mix was processed as described in fig 3.22. Lane '-', in all the gels contains RecA but no Ssb_{NC}. Substrate ssDNA and linear dsDNA are marked and product is marked as "NC" respectively. The substrates used for Deinococcal RecA were Ø174 ssDNA and linear Ø174 dsDNA, whereas for *E. coli* RecA the substrates used were M13ssDNA and linear M13dsDNA.

3.4.4. Strand exchange activity of Deinococcal or *E. coli* RecA using combination of N and C-terminal domains.

Strand exchange assay was performed with a combination of Ssb_N or Ssb_{NC} with Ssb_C. The combination of either Ssb_N or Ssb_{NC} with Ssb_C was made first, and then added to reaction mix in SS-DS or DS-SS reaction mode with Deinococcal RecA. In case of *E. coli* RecA, the reaction was carried out in SS-DS mode only. The combination of both Ssb_N/Ssb_C or combination of Ssb_{NC}/Ssb_C (Fig. 3.27) also did not affect nicked circular product formation with RecA from *Deinococcus* or *E. coli*.

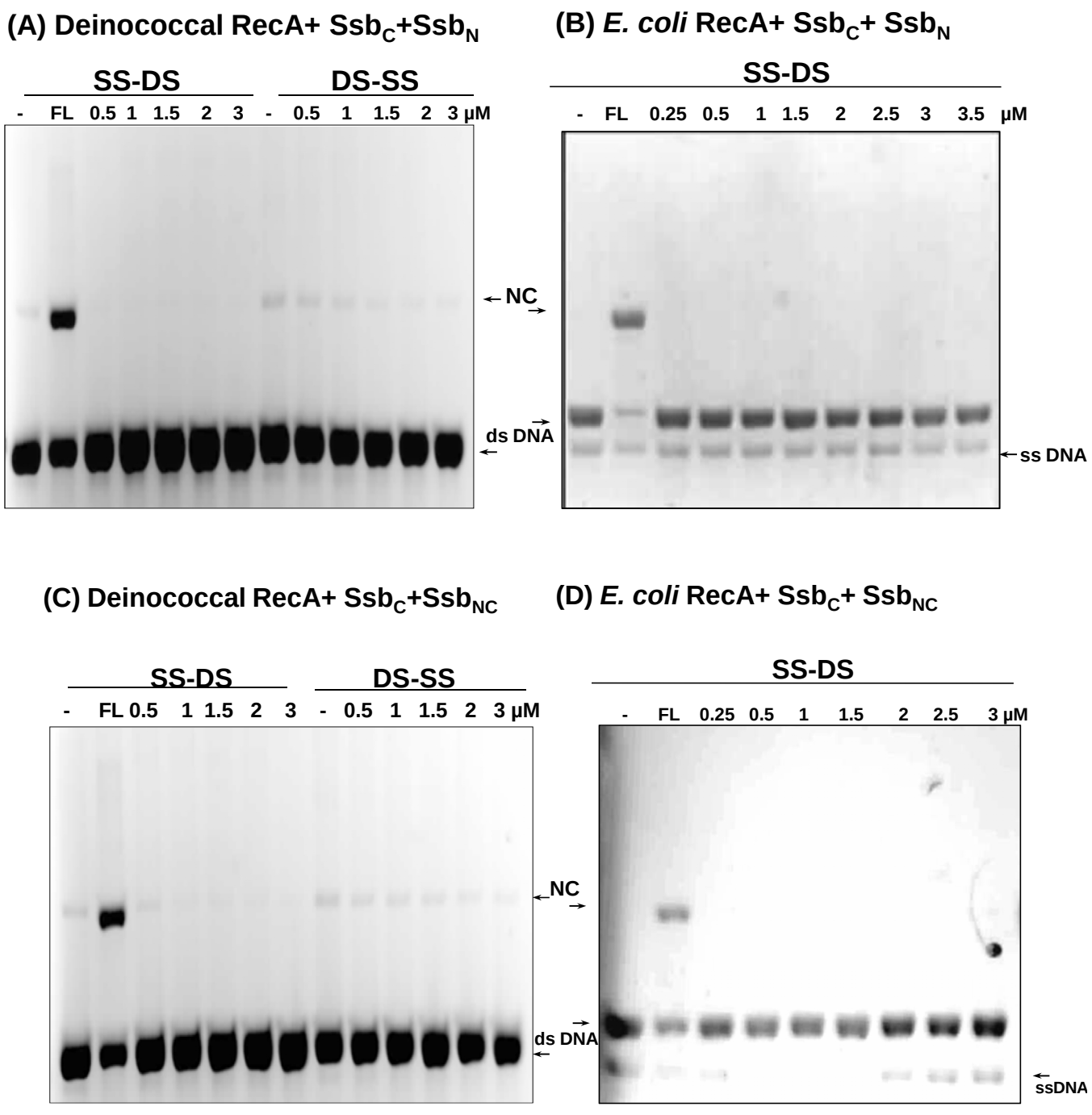


Fig. 3.27. DNA strand exchange assay with combination of N-terminal and C-terminal domains. Assay was performed with Deinococcal RecA in the presence of (A) Ssb_N+Ssb_C or (C) Ssb_{NC}+Ssb_C (at concentrations indicated on top of each lane) in SS-DS or DS-SS mode and *E. coli* RecA in the presence of (B) Ssb_N+Ssb_C and (D) Ssb_{NC}+Ssb_C in SS-DS mode. The reaction mix was processed as described in Fig 3.22. Lane "-", in all the gels contained RecA but no Ssb variants. Lane FL in (A), (B), (C) and (D) contained Ssb_{FL} (2 µM) as a positive control. Substrate ssDNA and linear dsDNA are marked and product is marked as "NC" respectively.

3.4.5. Titration of Ssb_N or Ssb_{NC} with fixed concentration of Ssb_C to enhance product formation in strand exchange assay

As reported previously in EMSA experiments that Ssb_N and Ssb_{NC} can individually interact physically and functionally with Ssb_C *in vitro*, and the complex displays a higher ssDNA binding activity, in comparison to the individual components (Fig 3.13). The experiment was done by keeping the concentration of Ssbc fixed at 2.5 µM and titration was carried out with increasing concentration of either Ssb_N or Ssb_{NC} (Fig. 3.28). In the SS-DS mode, there was some formation of intermediate joint molecule with *E. coli* RecA, which however did not convert into nicked circular molecule product. Deinococcal RecA activity was not enhanced in this assay and no joint molecule products were formed.

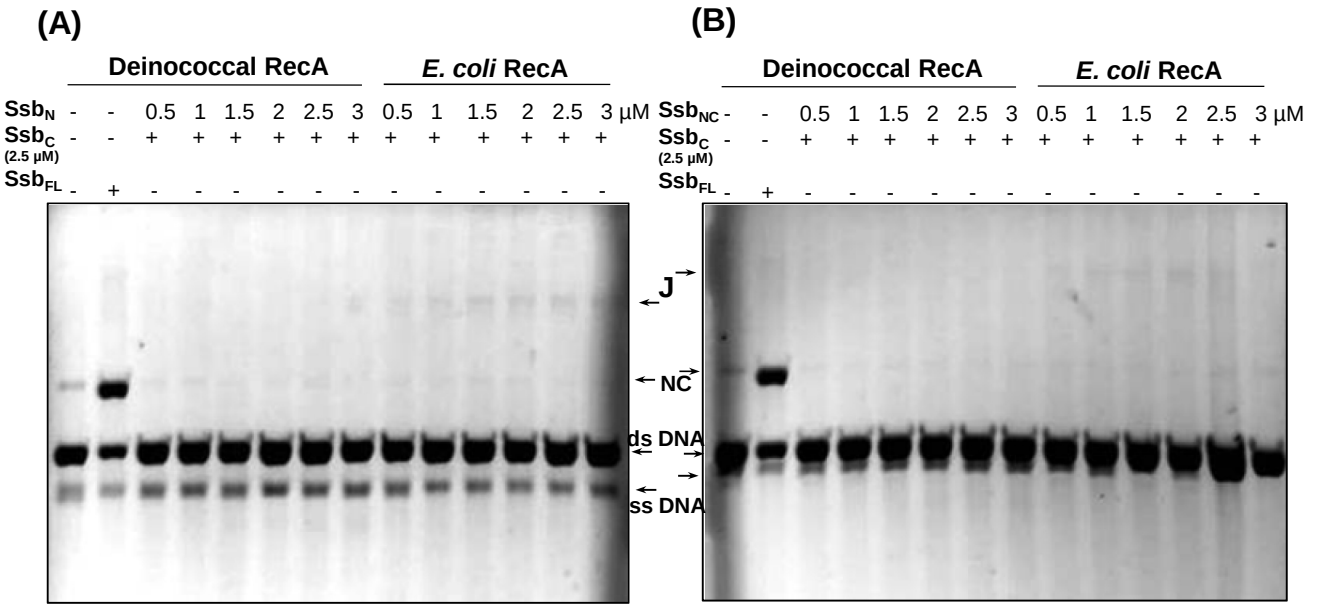


Fig. 3.28. DNA strand exchange assay performed with combination of N-terminal and C-terminal domains. Assay was carried out with Deinococcal RecA or *E. coli* RecA in the presence of (A) Ssb_C at fixed concentration of 2.5 µM with increasing concentration of Ssb_N or (B) Ssb_C at fixed concentration of 2.5 µM with increasing concentration of Ssb_{NC}. The concentrations of Ssb_N/Ssb_{NC} added in each lane is indicated on the top of each lane. Lane "-", in all the gels contained RecA but no Ssb variants. Ssb_{FL} (2 µM) was added as a positive control in lane 2 of figure (A) and (B). Substrate ssDNA and linear dsDNA are marked. Product and joint molecules formed are marked as "NC" and "J" respectively. Other details were described in Fig. 3.22.

3.4.6. Strand Exchange assay with combination of Ssb_N/Ssb_{NC} and Ssb_C, generated on Ni-NTA column.

As demonstrated previously by Ni-NTA affinity chromatography, Ssb_N or Ssb_{NC} interacted *in vitro* with Ssb_C. The combination protein complex, as obtained from Ni-NTA affinity chromatography (Fig. 3.15), was also used in strand exchange assay with Deinococcal RecA. The Ni-NTA generated column combination did generated intermediate joint molecules product which, however, did not convert into nicked circular molecule product (Fig. 3.29).

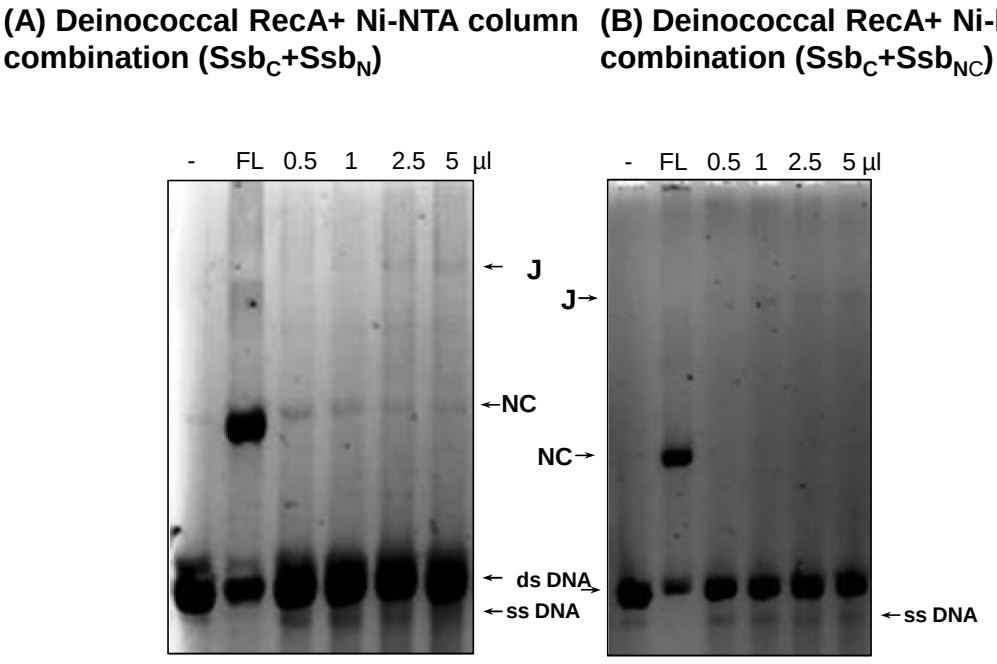


Fig. 3.29. DNA strand exchange assay performed with Ni-NTA column generated combination of N-terminal and C-terminal domains. Assay was carried out with Deinococcal RecA in the presence of (A) Ni-NTA generated combination of Ssb_C and Ssb_N (B) Ni-NTA generated combination of Ssb_C and Ssb_{NC}. The volume of Ssb_N/Ssb_{NC} and Ssb_C combination added in each lane is indicated on top of each lane. Lane "-", in all the gels contained RecA but no Ssb variants. Ssb_{FL} (2 µM) was added as a positive control in lane 2 of figure (A) and (B). Substrate ssDNA and linear dsDNA are marked. Product and joint molecules formed are marked as "NC" and "J" respectively. Other details were described in Fig. 3.22.

3.4.7. Strand exchange with glutaraldehyde cross-linked Ssb_N and Ssb_C

An attempt was made to obtain active artificially linked N-terminal and C-terminal domains by glutaraldehyde cross-linking, since the individual N and C-terminal domains as well as their *in vitro* combination were unable to assist Deinococcal or *E. coli* RecA in carrying out strand exchange. The cross-linked products were first checked for binding activity by EMSA with DIG labeled dT50 oligo. The binding activity was retained only when cross-linking agent was kept at a final concentration of 0.0125 and 0.03% (Fig. 3.30 A). Strand exchange assay was then performed with Deinococcal RecA and cross-linked Ssb_N/Ssb_C domains. Results showed that glutaraldehyde cross-linked product also did not enhance formation of nicked circular product molecules (Fig. 3.30. B, C).

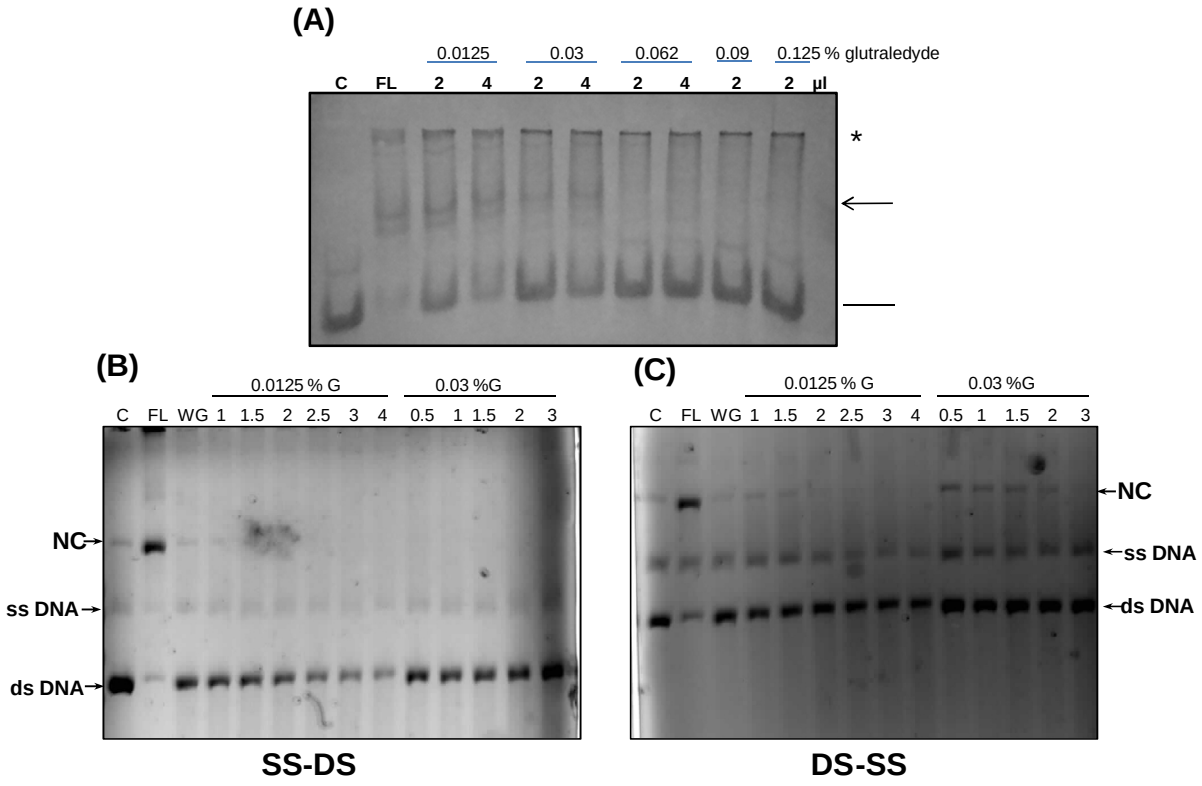


Fig. 3.30. DNA strand exchange assay performed with glutaraldehyde cross-linked combination of N-terminal and C-terminal domains. (A) EMSA of glutaraldehyde cross-linked Ssb_N and Ssb_C with dT50. The amount of glutaraldehyde used for cross-linking is indicated on top of each lane. The cross-linked products were dialyzed extensively to remove traces of cross-linking agent. DNA substrate and Ssb-protein complex are shown by "—" and "←", respectively while wells of the gels are marked by asterisk. (B) Strand exchange assay with cross-linked Ssb_N and Ssb_C in SS-DS or (C) DS-SS mode. The cross-linking was done at two indicated concentrations of glutaraldehyde. The volume of cross-linked Ssb_N and Ssb_C combination added in each lane is indicated at the top of the gel. Lane "-", in all the gels contains RecA but no Ssb variants. Ssb_{FL} (2 µM) was added as a positive control in lane 2 of figure (B) and (C). Lane WG contained non-treated protein as internal control. Substrate ssDNA and linear dsDNA are marked and product is marked as "NC" respectively. The reaction mix was processed as described in Fig. 3.22.

3.4.8. EMSA under strand exchange assay conditions.

As any of the Ssb variants, except Ssb_{FL}, could not enhance strand exchange activity of RecA, DNA binding activity of all the Ssb variants and their combinations was re-ascertained under strand exchange assay condition. EMSA was carried out under strand

exchange conditions and did result in the formation of prominent DNA-protein complexes of Ssb_{NC} or Ssb_N with Ssb_C (Figure 3.31), as observed earlier (Fig.3.13). Thus, the N-terminal and C-terminal Ssb variants did bind ssDNA and form the expected DNA-protein complexes even under strand exchange assay conditions, but were unable to remove secondary structures of virion ØX174 ssDNA required for strand exchange by RecA. Ssb_{FL} binds to ssDNA with highest affinity compared to all the variants of Ssb and is capable of removing such secondary structures. This appears to be since it contains N-terminal and C-terminal portions naturally linked by connector region, in the most optimal conformation necessary for enhancement of RecA mediated strand exchange activity.

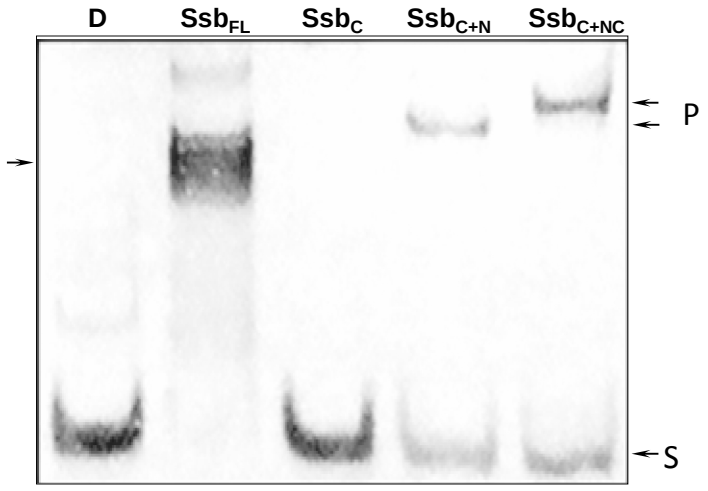


Fig. 3.31. EMSA carried out under strand exchange assay conditions. Lane 1 contained (0.125 µM) ssDNA, lane 2 contained Ssb_{FL} (0.5 µM), lane 3 contained Ssb_C (0.5 µM), lane 4 contained Ssb_C and Ssb_N (0.5 µM each) and lane 5 contained Ssb_C and Ssb_{NC} (0.5 µM) each. The substrate and product molecules are indicated by "S" and "P" respectively.

3.4.9. Effect of Ssb variants on the strand exchange promoting activity of Ssb_{FL} with Deinococcal RecA

Ssb_{FL} enhanced nicked circular product formation by RecA mediated strand exchange reaction (Fig. 3.23). Ssb_{FL} was mixed with Ssb_C or Ssb_N and then added in strand exchange reaction, to assess effect of individual Ssb domains, if any, on strand exchange activity promoted by Ssb_{FL}. Neither Ssb_C nor Ssb_N could inhibit Ssb_{FL} mediated enhancement of strand exchange reaction of Deinococcal RecA and no change in product formation was observed (Fig. 3.32).

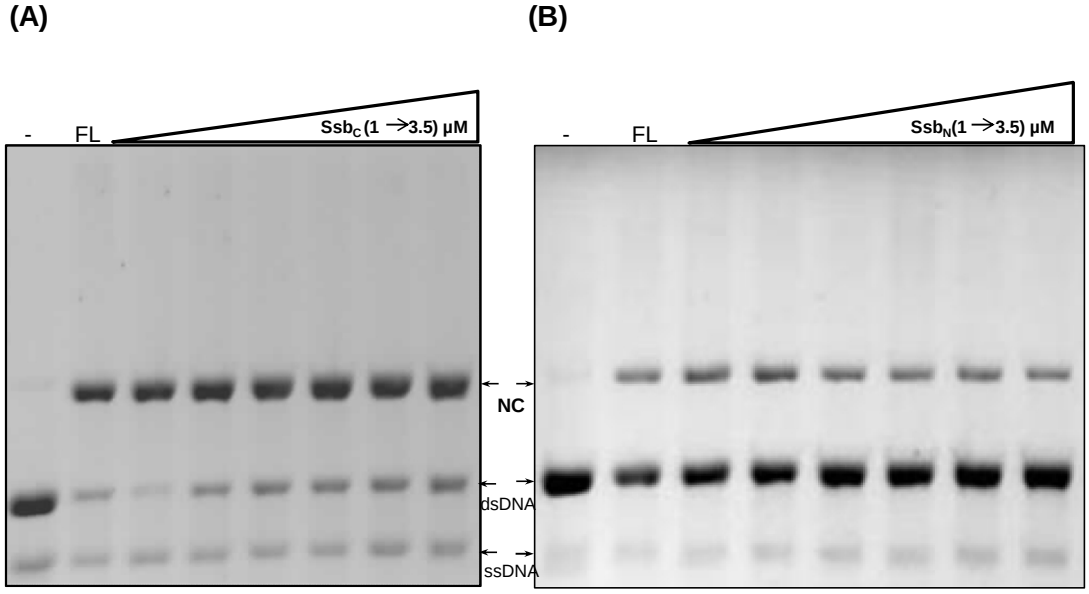


Fig. 3.32. DNA strand exchange assay performed with combination of full length Ssb and N-terminal or C-terminal domains. Assay was carried out with Deinococcal RecA in the presence of (A) Ssb_{FL} concentration fixed at 2 µM and increasing concentration of Ssb_C. (B) Ssb_{FL} concentration fixed at 2 µM and increasing concentration of Ssb_N. Lane "-", in both the gels contained RecA but no Ssb variants. Ssb_{FL} (2 µM) was added as a positive control in lane 2 of (A) and (B). Substrate ssDNA and linear dsDNA are marked and product is marked as "NC" respectively. The reaction mix was processed as described in Fig. 3.22.

In vitro studies clearly showed the differential activity of twin OB folds present in Deinococcal Ssb. As Ssb protein plays a major role in recombination and repair following DNA damage, the modulation in its levels was evaluated following different stresses, which lead to different intensity of DNA damage.

3.5. Modulation of Ssb levels following exposure to DNA damaging stressor.

Deinococcus radiodurans is known to survive extreme stress of oxidative and non-oxidative nature. It survives such stress using a highly efficient DNA repair system, comprising of several DNA repair proteins including Ssb. Ssb is an essential house keeping protein required in all aspects of DNA metabolism such as replication, recombination and repair. Ssb keeps DNA in single stranded form, so that other repair proteins can act upon it. It also protects single stranded DNA by coating the surface and resisting nucleolytic degradation.

DNA damaging stresses such as radiation, chemical mutagens and desiccation lead to enhanced expression of many DNA repair genes, which play a decisive role in survival and recovery of organisms. The expression profile of this important gene was therefore monitored under DNA damaging stresses of radiation, mitomycin C, desiccation, hydrogen peroxide and UV rays.

D. radiodurans survives up to 15 kGy dose of gamma (γ)-rays with 10 kGy as its D_{10} dose (which allows only 10% survival). γ-radiation causes direct and indirect (through induction of reactive oxygen species) damage to DNA. The extent of damage depends both on the dose as well as the dose rate. Ssb expression was therefore examined at a different doses imparted at high and low dose rates.

3.5.1. Effect of high dose rate γ-radiation doses on *ssb* gene expression.

The expression profile of *ssb* gene upon exposure to high dose rate gamma radiation stress was studied by applying different doses of radiation (1 to 8 kGy) at the fixed dose rate of 7.2 kGy/h.

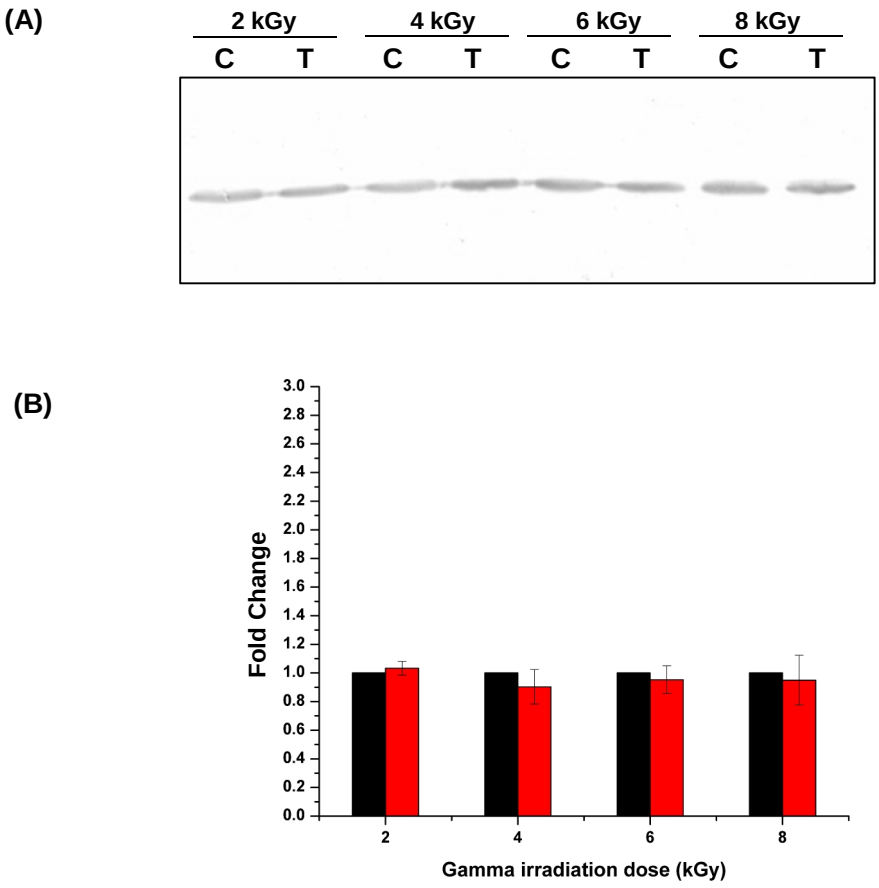


Fig. 3.33. Effect of γ-radiation doses imparted at high dose rate on *ssb* gene expression in *Deinococcus radiodurans*. Overnight grown deinococcal cells at final OD_{600}=5 were subjected to radiation stress of 2-8 kGy at the high dose rate of 7.2 kGy/hr. (A) Proteins were extracted at 2 h of PIR, resolved by 12% SDS-PAGE (30 µg/lane), blotted and probed by anti-DrSsb (1:200) and western blot developed using NBT/BCIP. (B) Graph showing mean values (of three independent experiments) of fold change in Ssb levels before (black) and following radiation stress (red). The error bars denote standard error of mean (SEM).

The samples were taken at 2h of PIR and Ssb expression was analyzed by western blotting of cell free extracts followed by immunodetection using a polyclonal antibody raised against *D. radiodurans* Ssb protein (anti-DrSsb). At such high dose rate, no significant induction in the levels of Ssb protein was observed. The experiment was repeated three times and a representative western blot is shown in Fig. 3.33.

3.5.2. Effect of low dose rate γ-radiation doses on *ssb* gene expression.

The expression profile of *ssb* gene was also studied by giving different doses of radiation (1 to 8 kGy) at a fixed low dose rate of 0.36 kGy/h. The samples were analyzed at 3h of PIR (Fig.3.34). Significant induction in the levels of Ssb protein was noticed at 3 to 8 kGy doses at this low dose rate. The highest expression was observed at 7 kGy. The representative western blot depicting radiation enhanced *ssb* expression is shown in Fig 3.34.

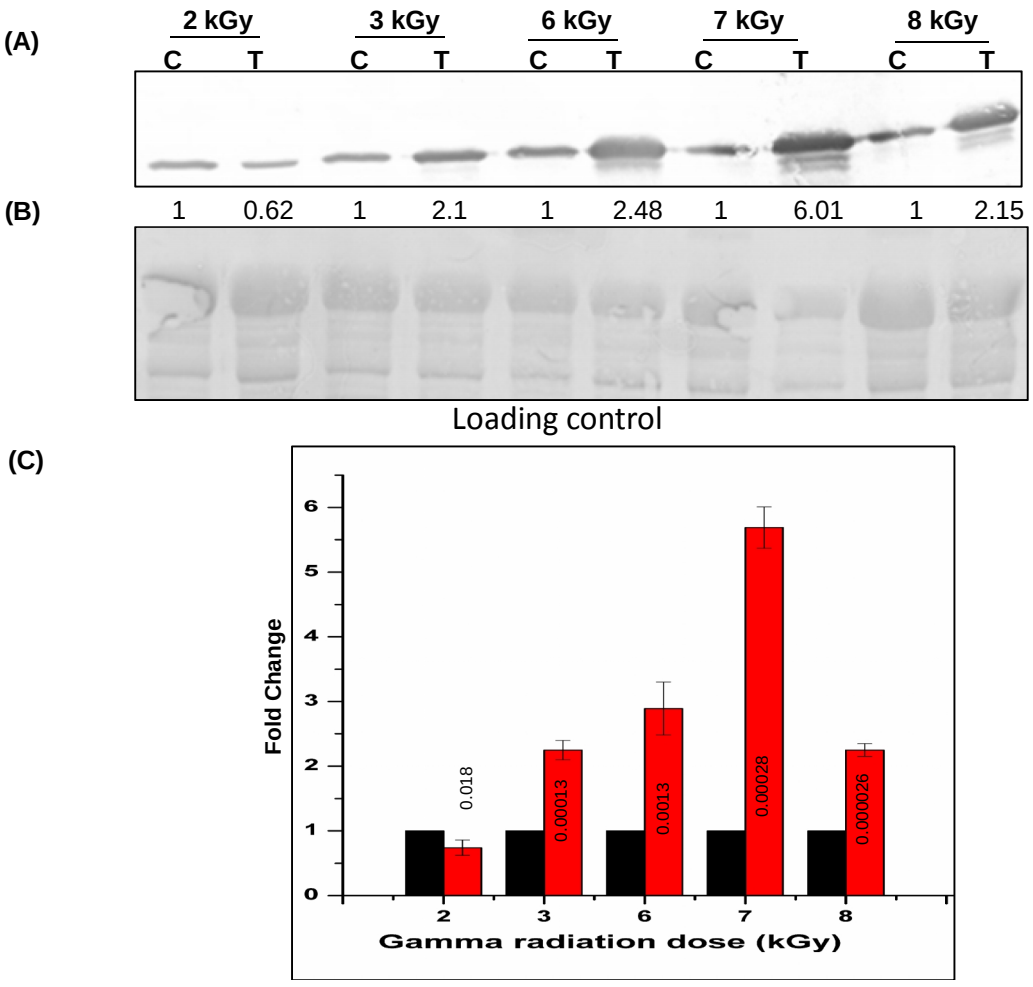


Fig. 3.34. Effect of γ-radiation doses imparted at low dose rate on *ssb* gene expression in *Deinococcus radiodurans*. Overnight grown deinococcal cells at final OD_{600}=5 were subjected to radiation stress of 2-8 kGy at the low dose rate of 0.36 kGy/hr. The cells were subsequently washed and allowed to recover in fresh TGY medium for 3h. Cellular proteins were extracted, resolved by 12% SDS-PAGE (100 µg/lane), blotted and probed by anti-DrSsb antibody and visualized using NBT/BCIP. (A) A representative western blot is shown. The numbers below the lanes depict quantitation of Ssb levels by densitometry (B) Corresponding loading control resolved by 12% SDS-PAGE and stained with CBB. (C) Graph showing mean values (of three independent experiments) of fold change in Ssb levels before (black) and following radiation stress (red). The error bars denote standard error of mean (SEM). The p value is indicated along bars for each dose.

3.5.2. Effect of 7 kGy radiation dose on the post irradiation recovery of *Deinococcus radiodurans*.

Since optimal induction in Ssb levels was seen at 7 kGy (Fig. 3.34), the post irradiation recovery (PIR) of cells after 7 kGy of irradiation was monitored by estimating turbidity (OD at 600nm), (A) and also simultaneously by plate count (B). After 7 kGy dose delivered at 0.36 kGy/h, the cells entered into a lag phase of 3 h, after which they resumed active growth (Fig 3.35).

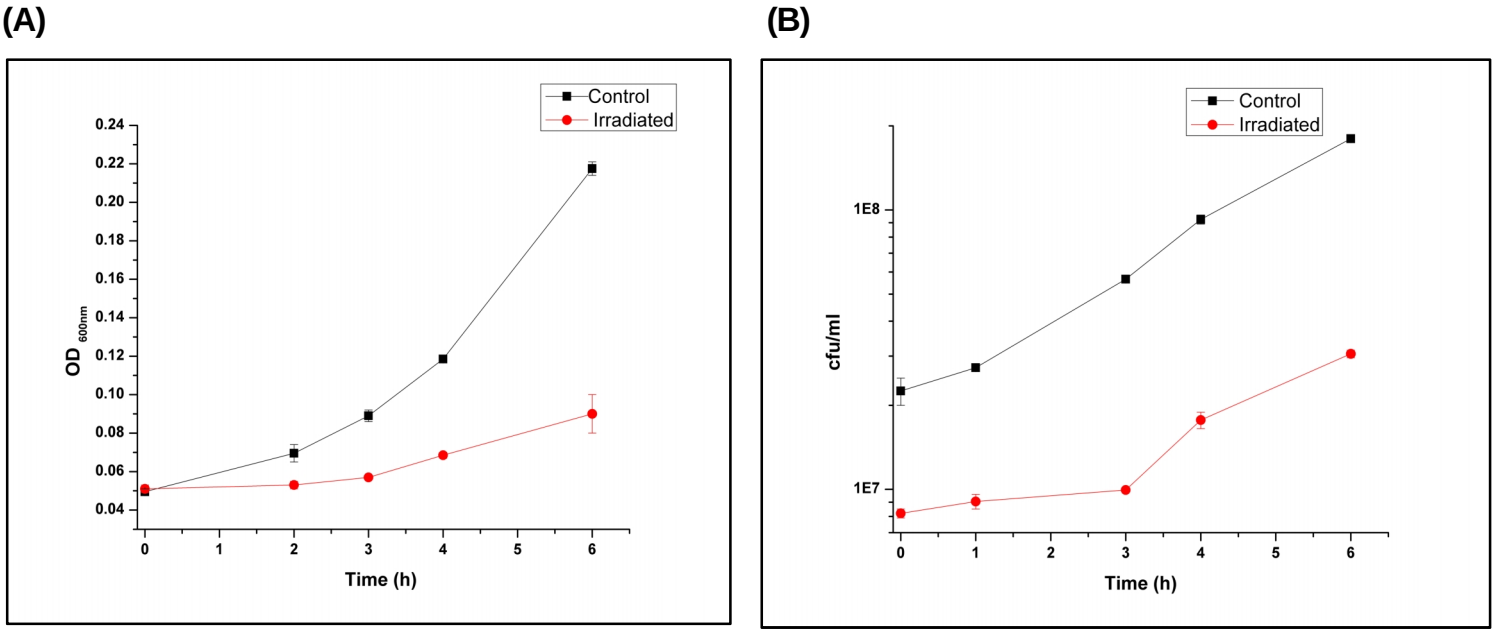


Fig. 3.35. Effect of 7 kGy radiation dose on the post-irradiation recovery of *Deinococcus radiodurans*. Cells were subjected to 7 kGy radiation dose at a dose rate of 0.36 kGy and were allowed to recover for 6 h. (A) OD at 600nm was taken at the indicated time intervals (B) colony forming units estimated at corresponding time points, shown as a semi log plot.

3.5.4. Time course of *ssb* gene expression after 7 kGy dose of γ-rays.

As described earlier, at low dose rate, the *ssb* gene expression increased according to increment in radiation dose, with maximum induction obtained at 7kGy. At this dose of 7 kGy at 0.36 kGy/h dose rate, the expression profile was studied at different time points of post irradiation recovery (Fig. 3.36).

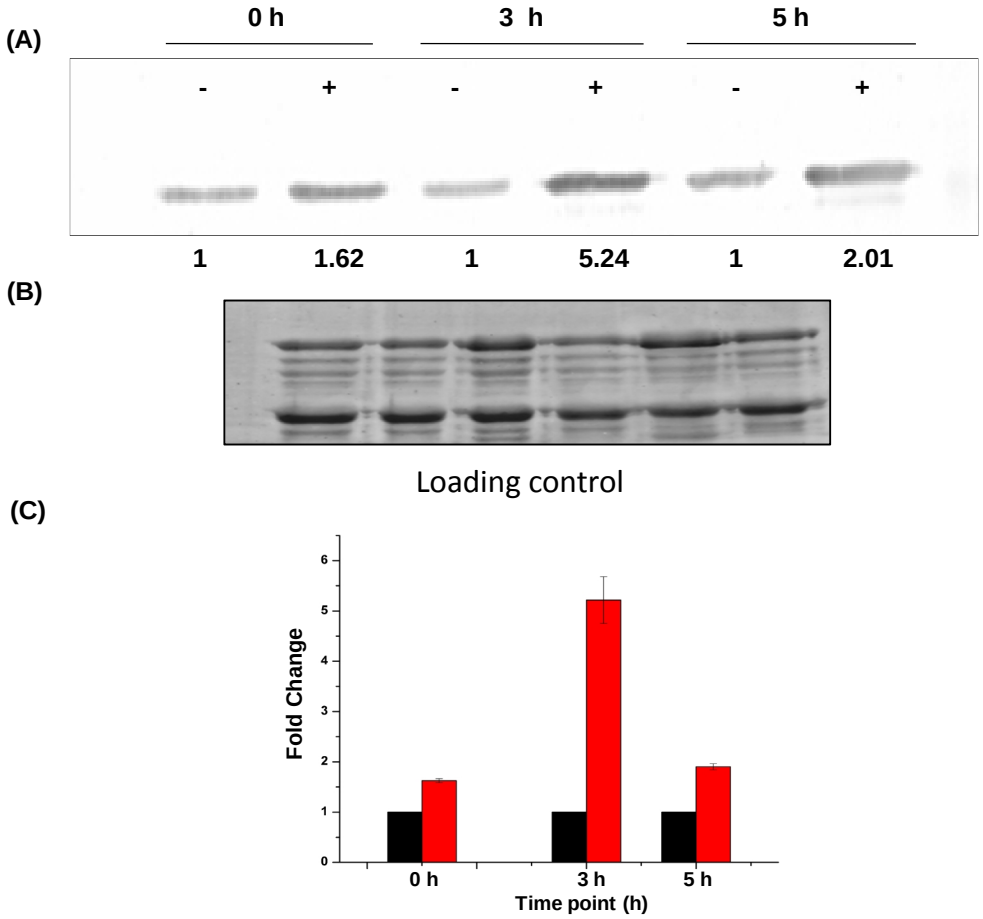


Fig. 3.36. Time course of *ssb* gene expression during PIR. About 30 µg protein/lane at indicated time interval was resolved by 12% SDS-PAGE, blotted and probed by anti-DrSsb antibody and visualized using NBT/BCIP. (A) Representative western blot of Ssb profile obtained at different time points of post irradiation recovery after 7 kGy dose imparted at 0.36 kGy/h. The numbers below the lanes depict quantitation of Ssb levels by densitometry (B) Corresponding loading control resolved by 12% SDS-PAGE and stained with Coomassie brilliant blue dye. (C) Graph showing mean values (of three independent experiments) of fold change in Ssb levels at different time points before (black) and following radiation stress (red). The error bars denote standard error of mean (SEM).

A significant induction in the levels of Ssb was observed and the expression levels were maximal at 3 h of post-stress recovery, after which the Ssb levels declined. Major DNA repair in *Deinococcus* occurs in this growth arrest phase and involves initial RecA independent and subsequently RecA dependant pathways. The induction in Ssb levels signifies its importance in DNA repair in *D. radiodurans* along with other repair proteins. Proteomic data from this laboratory has also demonstrated rapid induction and processing of Ssb during initial phase of PIR in *D. radiodurans* [163].

3.5.5. Post-irradiation transcriptional induction of *ssb*.

The mechanism underlying radiation induced *ssb* gene expression was investigated. The cells were incubated with 50 µg/ml of rifampicin prior to irradiation or during post-irradiation recovery, with their corresponding controls, and Ssb levels were immuno-detected with anti-DrSsb antibody. Significant induction in levels of Ssb was observed in irradiated samples as well as in samples which had rifampicin added during irradiation but washed off during recovery. In contrast, the induction levels dropped significantly in the irradiated samples wherein rifampicin was added during recovery. Samples which had rifampicin during irradiation as well as recovery also did not show any induction in Ssb levels. The results clearly showed that *ssb* gene expression is transcriptionally activated upon irradiation (Fig 3.37).

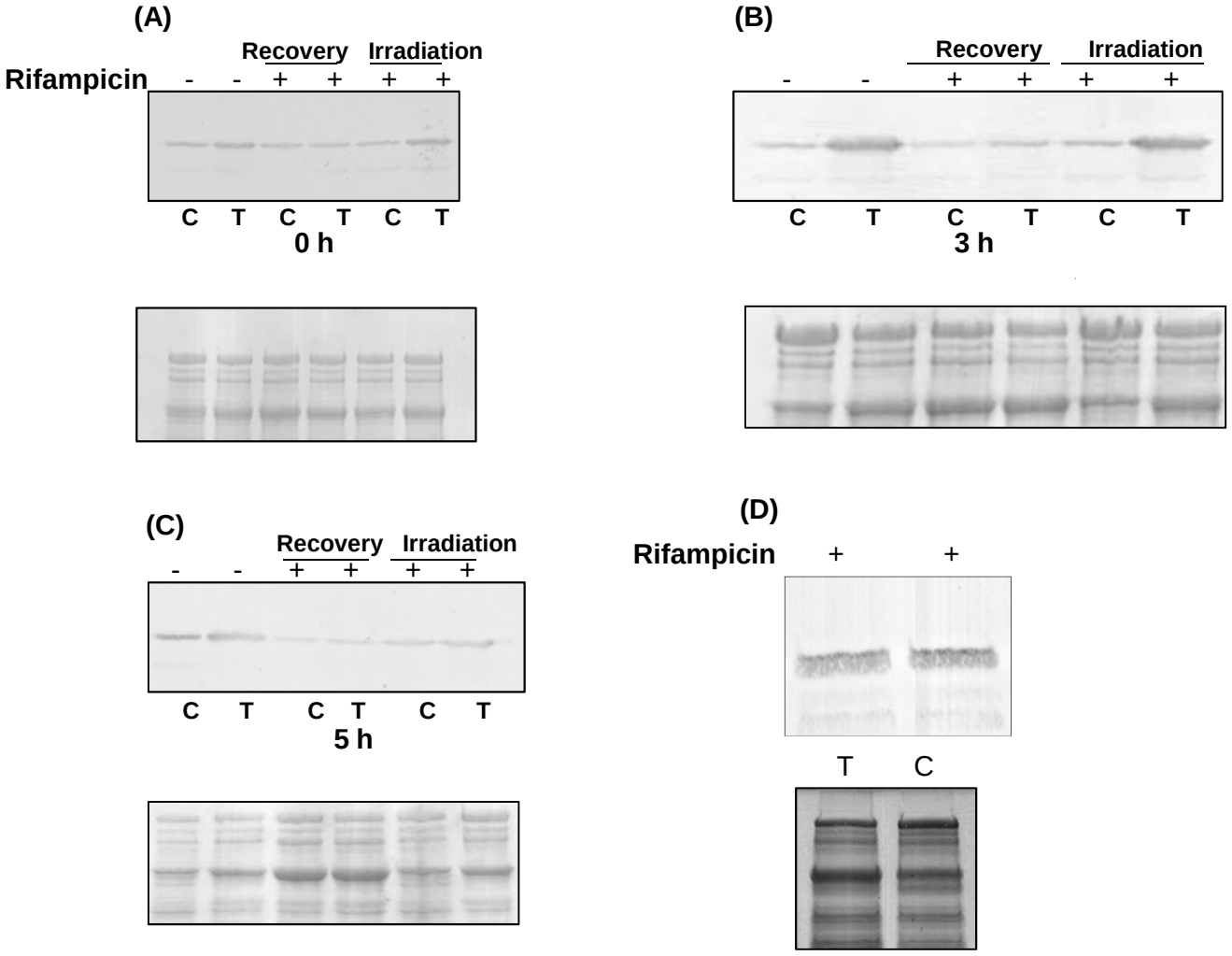


Fig. 3.37. Transcriptional induction of *ssb* gene in *D. radiodurans*. Stationary-phase *D. radiodurans* cells were exposed to 7 kGy of γ-rays at 0.36 kGy/h dose rate in the presence of 50 µg/ ml of rifampicin, which was added either during PIR [lanes 3 and 4 of (A-C)] or added during irradiation and washed off before recovery [lanes 5 and 6 of (A-C)], or added during irradiated (7 kGy) and PIR (3 hrs) (lane 1 of D), and compared with unirradiated and irradiated cells [lanes 1 and 2 of (A-C)]. Post treatment cells were allowed to recover in TGY medium for three different time points of 0 (A), 3h(B) and 5h (C). Protein extracts (30 µg) were electrophoretically resolved, blotted and probed using anti-DrSsb antibody. The lanes in which rifampicin was added are indicated by "+".

3.5.6. Effect of mitomycin-C on *ssb* gene expression.

The well known mutagen mitomycin-C causes strand breakages and cross-linking of DNA resulting in formation of DNA adducts. In order to see if effect of radiation was a generic effect of DNA damage or specific to γ-rays, effect of mitomycin-C on *ssb* expression was investigated in *D. radiodurans*.

Deinococcal cells were exposed to 20μg/ml mitomycin-C for 25 mins. Aliquotes were withdrawn at indicated time intervals to assess the viability of cells by plating on TGY agar and determining colony forming units (CFU). The survival reduced to 40%-30% after exposure to mitomycin-C for 15 mins (Fig 3.38).

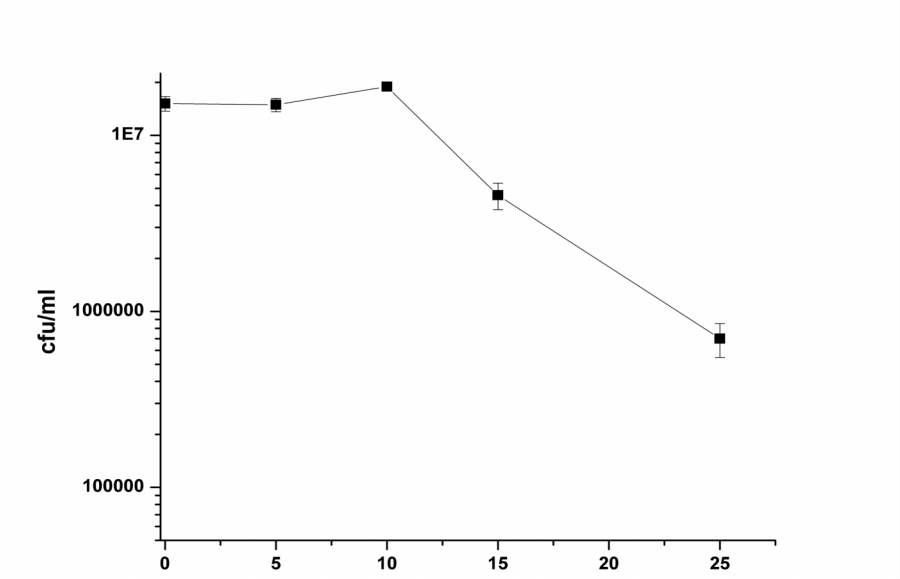


Fig. 3.38. Standardization of exposure time to Mitomycin-C in *Deinococcus* cells. Cells at OD_{600nm} of 5 were subjected to Mitomycin-C treatment for 25 mins at a final concentration of 20 μg/ml. Aliquotes were taken at indicated time points and plated on TGY agar to estimate survival as CFUs.

Mitomycin-C causes formation of DNA adducts when cells are exposured to this mutagen and leads to DNA breaks when replication, repair or recombination is carried out by various proteins [173]. Significant induction in the level of Ssb expression occurred upon exposure

to mitomycin-C. The induction pattern was similar to that obtained for radiation stress. The highest levels were recorded at 3 h of post-stress recovery, after which the Ssb levels declined (Fig. 3.39).

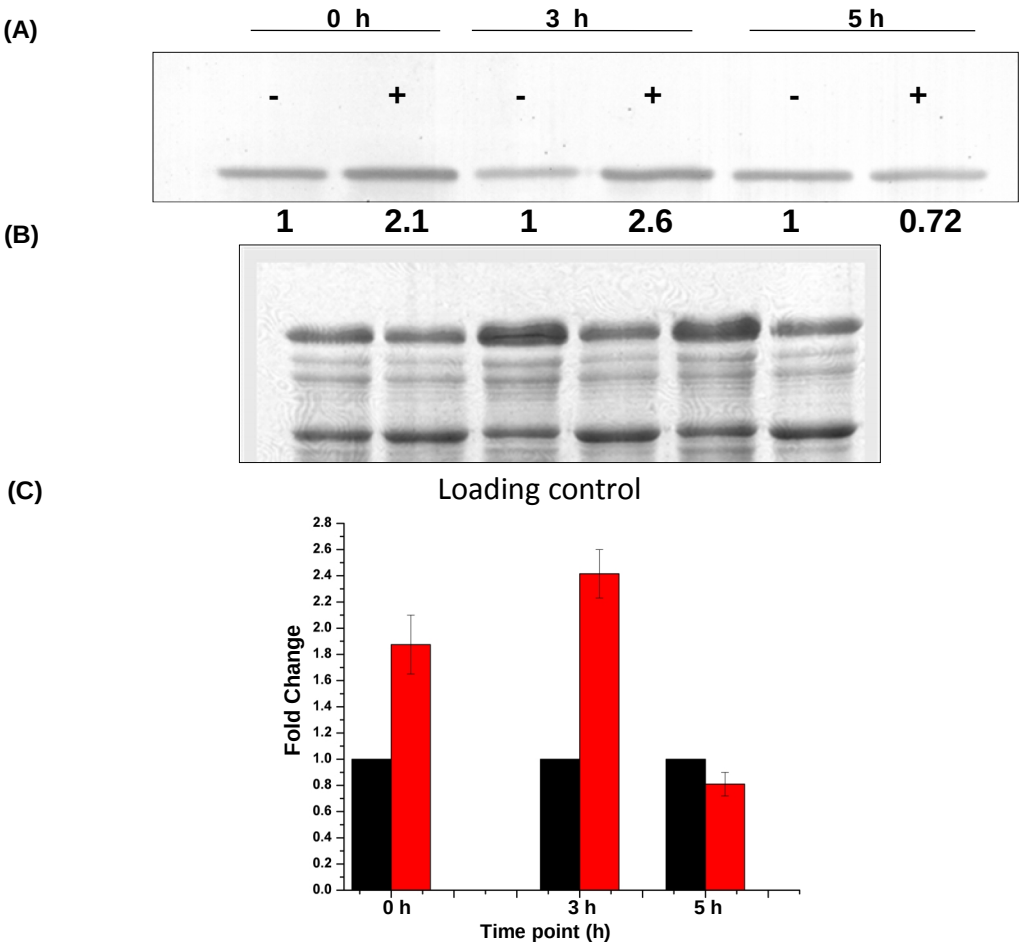


Fig. 3.39. Time course of *ssb* gene expression following Mitomycin-C stress. Overnight grown deinococcal cells at final OD 5 were subjected to Mitomycin-C stress of 20 µg/ml for 15 mins. The cells were subsequently washed and allowed to recover in fresh TGY medium for 5 h. About 30 µg protein/lane was resolved by 12% SDS-PAGE and probed as described in Fig. 3.36. (A) Representative western blot of Ssb profile obtained at different time points of post-stress recovery after exposure to 20 µg/ml of mitomycin-C for 15 mins. The numbers below the lanes depict quantitation of Ssb levels by densitometry (B) Corresponding loading control resolved by 12% SDS-PAGE and stained with Coomassie Brilliant Blue dye. (C) Graph showing mean values (of three independent experiments) of fold change in Ssb levels at different time points before (black) and following mitomycin-C stress (red). The error bars denote standard error of mean (SEM).

There was significant induction in *ssb* gene levels following radiation and mitomycin-C treatments, both of which lead to massive DNA damage in the form of double strand breaks (DSBs), single strand breaks (SSBs), individual base damage and DNA adducts. The *ssb* gene expression profile was also evaluated following lesser damaging stressors such as hydrogen per-oxide or UV.

Hydrogen per-oxide mainly generates reactive oxygen species (ROS), which primarily contribute to base and nucleotide damage and generation of few DNA strand breaks depending on the concentration of resident Fe^{2+} concentration present in bacterial cells.

3.5.7. Effect of hydrogen peroxide on *ssb* gene expression.

The survival curve of *D. radiodurans* in response to different concentrations of hydrogen peroxide stress is shown in Fig. 3.40. Close to 50% mortality was observed when Deinococcal cells were exposed to 50 mM hydrogen peroxide for 1 h.

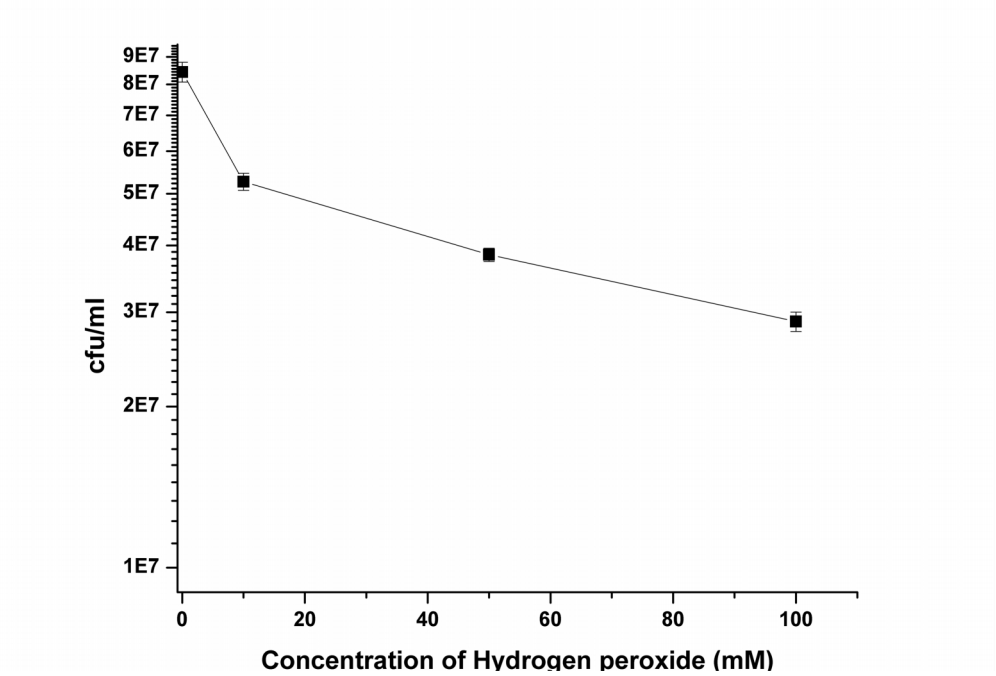


Fig. 3.40. Survival curve of *Deinococcus radiodurans* in response to hydrogen peroxide stress. Cells at OD_{600}=5 density were subjected to indicated concentration of hydrogen peroxide stress for 1 h. Following stress the cells were dilution plated on TGY agar plates to determine survival.

Exposure to hydrogen peroxide leads to free radical generation [174] which in turn causes considerable indirect DNA damage but relatively lesser DNA double strand breaks. No significant induction in Ssb protein levels were observed upon exposure to hydrogen peroxide (Fig. 3.41) signifying that DNA double strand breaks is a likely trigger for enhancement of Ssb transcription.

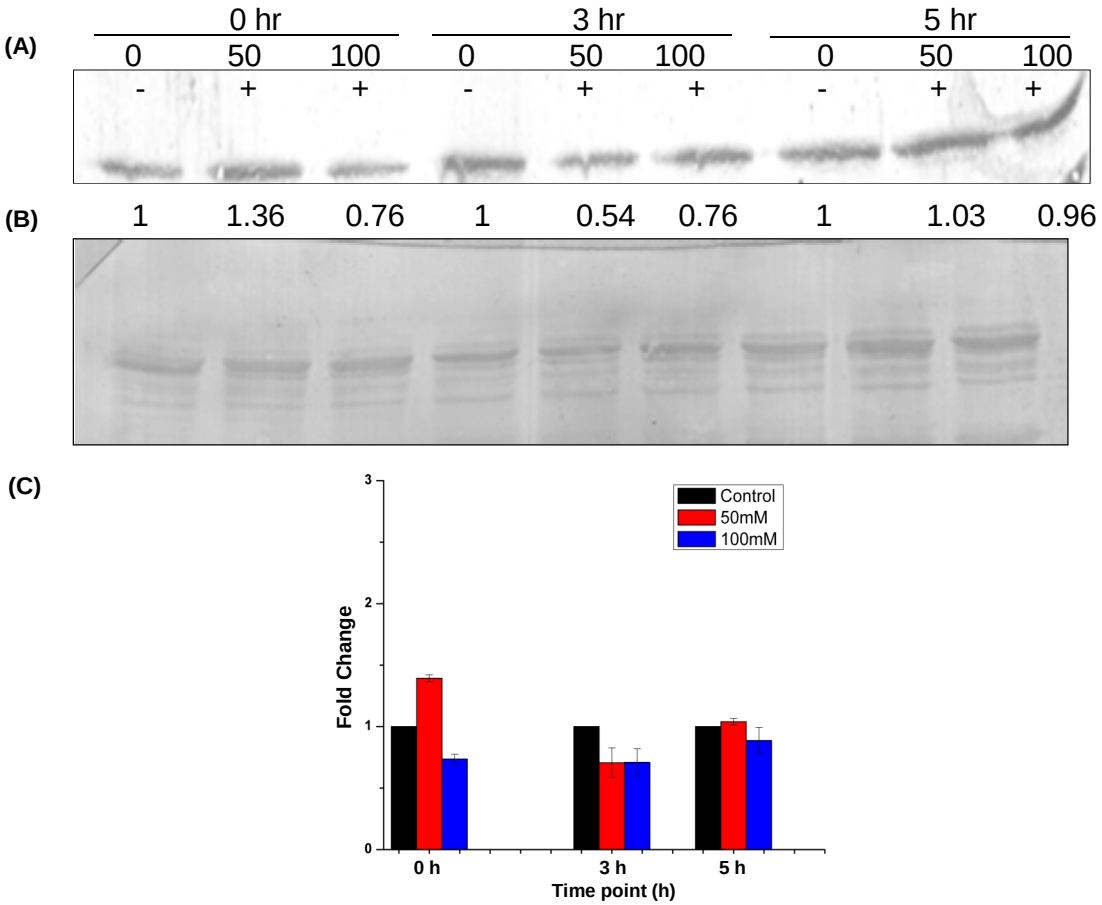


Fig. 3.41. Time course of *ssb* gene expression following hydrogen peroxide stress. Overnight grown deinococcal cells at final OD_{600}=5 were subjected to hydrogen peroxide stress of 50 mM and 100 mM for 1 h. The cells were subsequently washed and allowed to recover in fresh TGY medium for 5 h. About 30 µg proteins / lane was resolved by 12% SDS-PAGE and probed as described in Fig.3.36. (A) Representative western blot of Ssb profile obtained at different time points of post hydrogen peroxide recovery. The numbers below the lanes depict quantitation of Ssb levels by densitometry (B) Corresponding loading control resolved by 12% SDS-PAGE and stained with Coomassie Brilliant Blue dye. (C) Graph showing mean values (of three independent experiments) of fold change in Ssb levels at different time points before (black) and following hydrogen peroxide stress (red, 50 mM and blue 100 mM). The error bars denote standard error of mean (SEM).

3.5.8. Effect of non-ionizing UV radiation on *ssb* gene expression.

Non-ionizing radiations such as UV rays exhibit mutagenic effects by exciting the molecules present in DNA, leading to formation of extra new bonds in pyrimidines and resulting in formation of dimers (mostly thymidine dimers). Pyrimidine dimers affect shape of DNA and inhibit replication. The cell tries to remove dimers with the help of various enzymes but such repair may result in more mutations. The pyrimidine dimers are repaired by nucleotide excision repair pathways. Occasionally two pyrimidine dimers close to each other on DNA strand may lead to double strand breaks.

The survival curve for *D. radiodurans* in response to different doses of UV stress is shown in Fig. 3.42. Close to 50% mortality was observed when Deinococcal cells were exposed to UV dose of 2 kJm^{-2}.

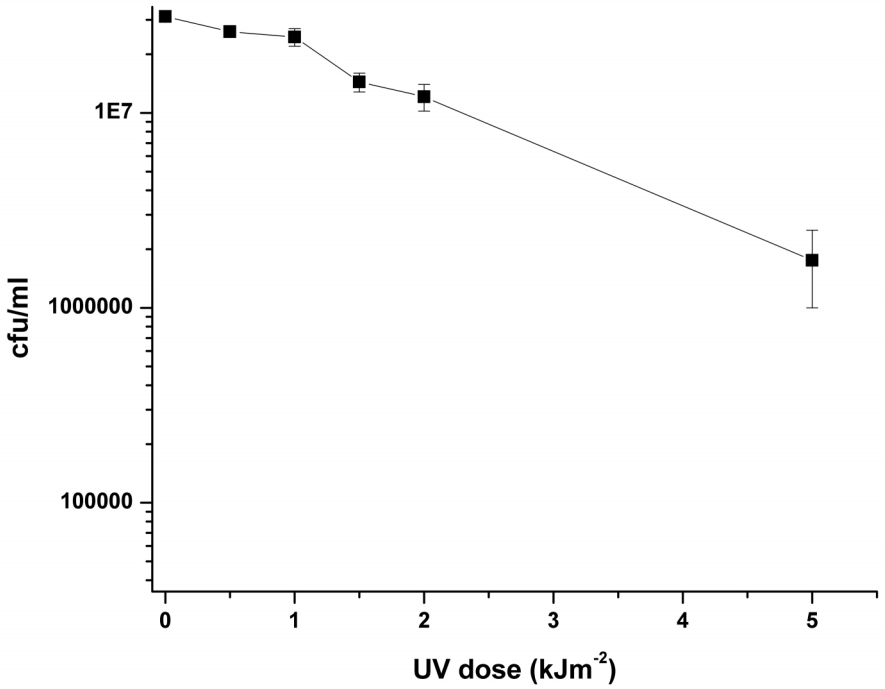


Fig. 3.42. Survival curve of *Deinococcus radiodurans* in response to UV radiation stress. *Deinococcus radiodurans* cells at OD_{600} =5 were subjected to UV radiation stress at the indicated doses. Following stress the cells were dilution plated on TGY agar plates to determine survival.

Notwithstanding the fact, the *Deinococcus* lacks photo reactivation repair, it exhibits a high tolerance to UV radiation. In line with this, no significant induction in Ssb protein levels was observed upon exposure of *D. radiodurans* cells to UV radiation (Fig. 3.43), signifying that DNA strand breaks is indeed a trigger for *ssb* induction.

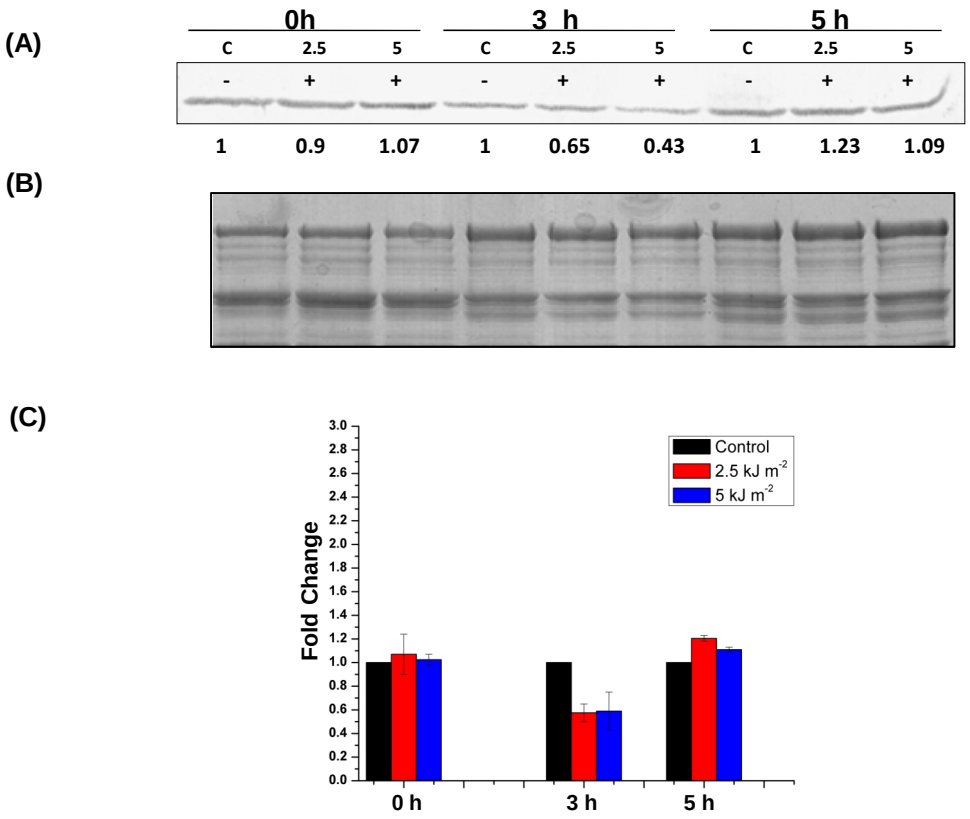


Fig. 3.43. Time course of *ssb* gene expression following UV radiation stress. Overnight grown deinococcal cells at final OD_{600}=5 were subjected to UV radiation stress of 2.5 and 5 kJm^{-2}. The cells were subsequently washed and allowed to recover in fresh TGY medium for 5 h. About 30 µg proteins/lane was resolved by 12% SDS-PAGE and probed as described in Fig.3.36. (A) Representative western blot of Ssb profile obtained at different time points of post UV stress recovery. The numbers below the lanes depict quantitation of Ssb levels by densitometry (B) Corresponding loading control resolved by 12% SDS-PAGE and stained with Coomassie Brilliant Blue dye. (C) Graph showing mean values (of three independent experiments) of fold change in Ssb levels at different time points before (black) and following UV stress (red, 2.5 kJm^{-2} and blue, 5 kJm^{-2}). The error bars denote standard error of mean (SEM).

3.5.9. Effect of desiccation on *ssb* gene expression.

The effect of desiccation stress is believed to be similar to γ-radiation stress. It also causes DNA damage similar to γ-radiation. It has also been speculated that resistance of *Deinococcus* towards gamma irradiation stress may be incidental and is likely due to adaptation of bacteria to desiccation stress. As desiccation stress involves long treatment time as compared to other studied stresses, conditions for desiccation needed to be standardized. Four types of controls were experimented with (a) cells deposited on membrane and CFUs determined without any storage, (b) cells deposited on membrane and stored on agar plate for the same duration as for desiccated samples, (c) cells deposited on membrane and stored under humid conditions for the same duration as for desiccated samples, and (d) cells deposited on membrane and stored in desiccator without desiccant for the same duration as for desiccated samples. After 7 days of treatment, CFUs were determined in all controls and the desiccated sample. The data are presented below in table.

Table 3.2. Survival of *D. radiodurans* during desiccation.

Post Desiccation recovery time	Control (a)	Agar control (b)	Humid control (c)	Dry control (d)	Desiccated (fused calcium chloride)
1 hr	1.64×10^7	7.4×10^6	7×10^5	0	1.76×10^7

The control cells (without treatment) were separated from the same batch of cells which were put for desiccation and immediately put to recovery for determining CFUs and was

subsequently used in experiments. Since in all other controls (b), (c) or (d) survival was adversely affected, the control shown in (a) (Table 3.1) was used in all further experiments.

The survival curve for *D. radiodurans* exposed to prolonged desiccation stress is shown in Fig. 3.44. Only 25% lethality was observed when cells were exposed to 6 weeks of desiccation stress.

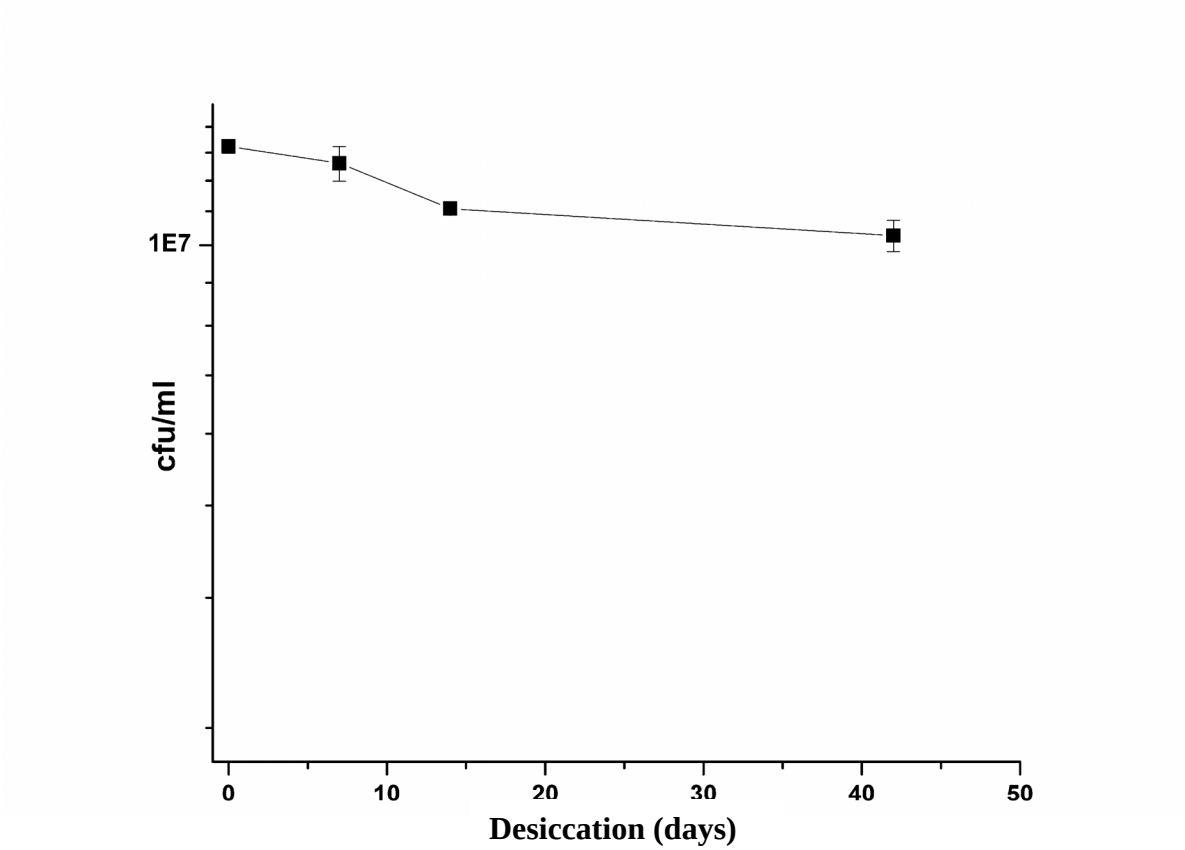


Fig. 3.44. Survival curve of *Deinococcus radiodurans* in response to desiccation stress. Cells at OD_{600nm}=5/ml were subjected desiccation stress upto 6 weeks. Following stress the cells were dilution plated on TGY agar plates to assess the CFUs. The survivability was compared with control cells.

It is known that the DNA damage by radiation and desiccation are similar, as both lead to similar DNA damage including double strand breaks. However, no significant induction in levels of Ssb after 1, 2 or 6 weeks of desiccation was oberved (Fig 3.45).

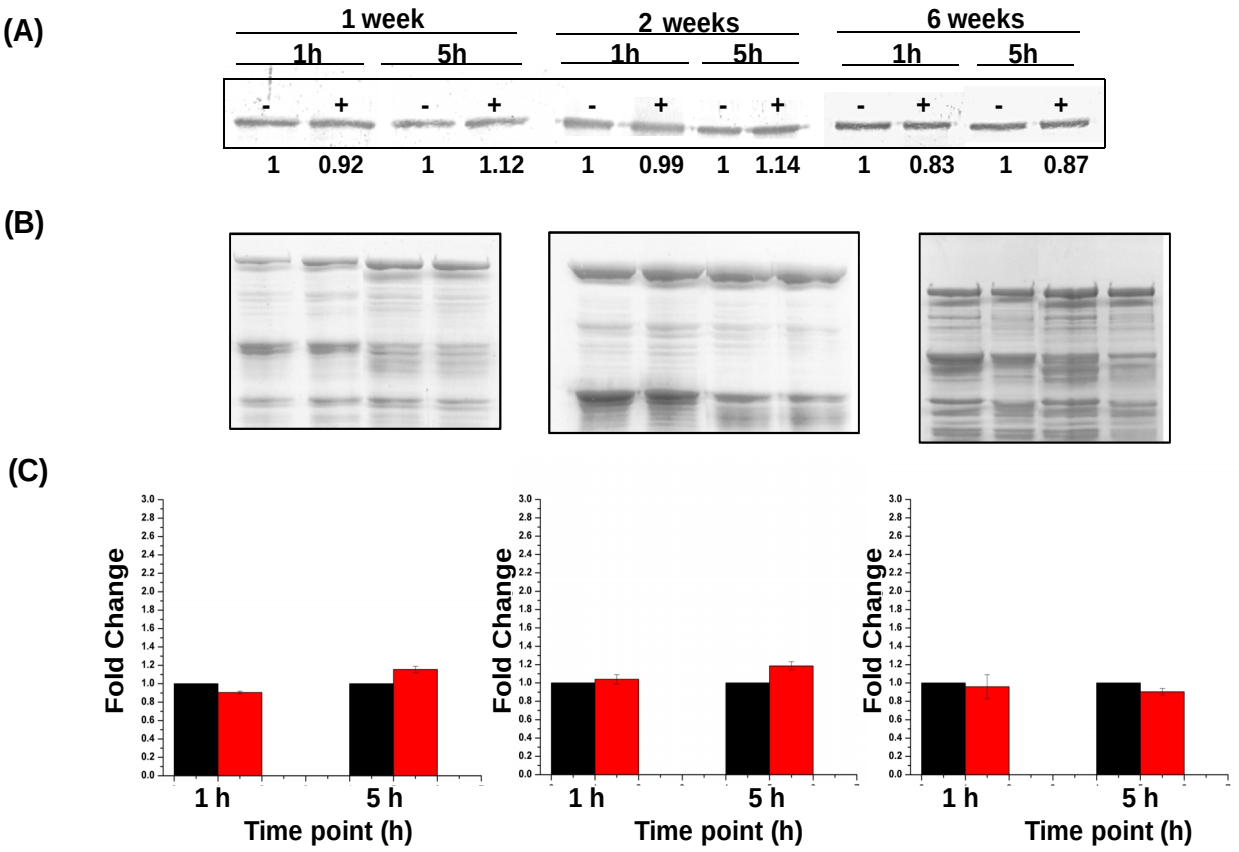


Fig. 3.45. Time course of *ssb* gene expression following desiccation stress. Overnight grown deinococcal cells at final OD_{600}=5/ml were subjected to desiccation stress of 1, 2 and 6 weeks. The cells were subsequently washed and allowed to recover in fresh TGY medium for 5 h. About 30 µg proteins / lane was resolved by 12% SDS-PAGE and probed as described in Fig.3.36. (A) Representative western blot of Ssb profile obtained at different time points during post desiccation stress recovery. The numbers below the lanes depict quantitation of Ssb levels by densitometry (B) Corresponding loading control resolved by 12% SDS-PAGE and stained with Coomassie brilliant blue dye. (C) Graph showing mean values (of three independent experiment) of fold change in Ssb levels at different time points of different weeks before (black) and following post desiccation stress recovery (red). The error bar denotes standard error of mean (SEM).

3.5.10. Effect of desiccation on *rec*A gene expression by western blotting.

RecA plays an important role in post-stress DNA recombination and repair, in two vital processes of ESDSA and homologous recombination. As desiccation and radiation are said to cause similar damage to DNA integrity and *rec*A induction following radiation is already established [175, 176], the *rec*A gene expression was also investigated following 2 and 6 weeks of desiccation.

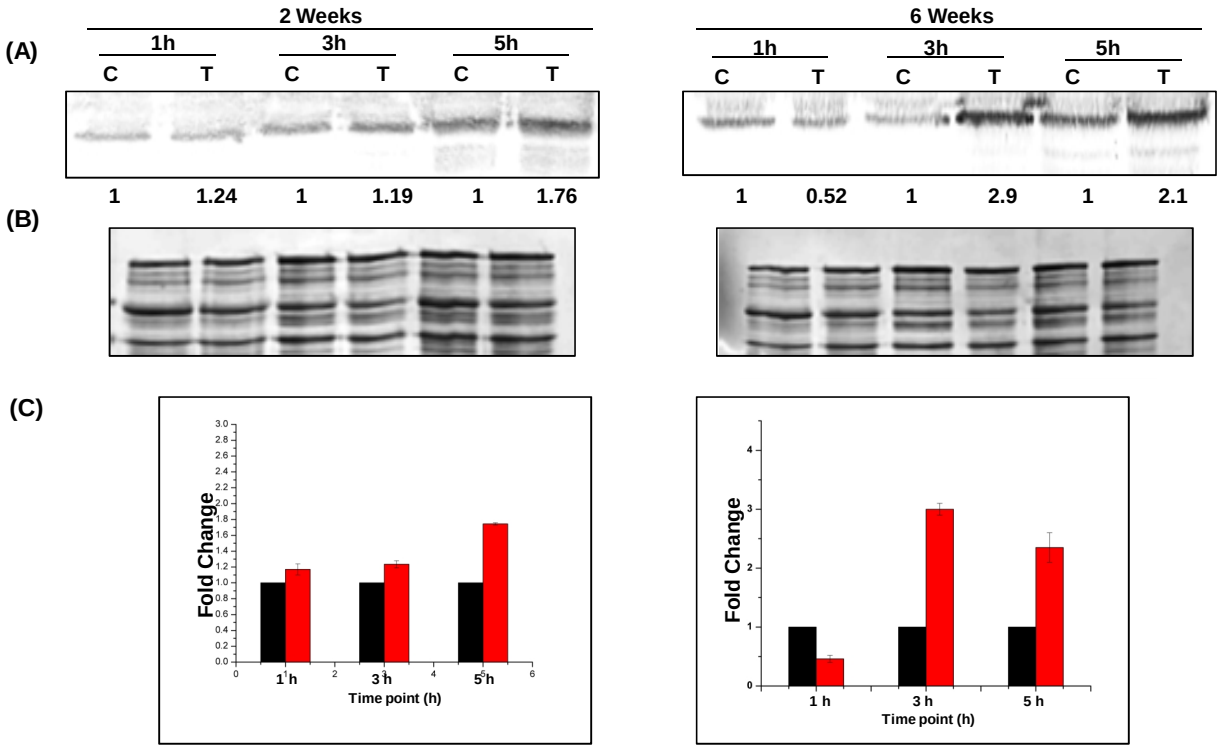


Fig. 3.46. Time course of *rec*A gene expression profile following desiccation stress. Overnight grown deinococcal cells at final OD_{600}=5/ml were subjected to desiccation stress of 2 and 6 weeks. The cells were subsequently washed and allowed to recover in fresh TGY medium for 5 h. The extracted proteins (30 µg/ lane) were resolved by 12% SDS-PAGE, probed by anti-RecA antibody (1:500 dilution) and the western blot developed using NBT/BCIP. (A) Representative western blot of RecA profile obtained at different time points during recovery following 2 and 6 weeks of desiccation. The numbers below the lanes depict quantitation of RecA levels by densitometry (B) Corresponding loading control resolved by 12% SDS-PAGE and stained with Coomassie Brilliant Blue dye. (C) Graph showing mean values (of three independent experiments) of fold change in RecA levels at different time points of different weeks before (black) and following post desiccation stress recovery (red). The error bars denote standard error of mean (SEM).

Unlike Ssb, significant induction in levels of RecA was found after 2 and 6 weeks of desiccation of Deinococcal cells. The induction was markedly seen after 3 h of post desiccation recovery. The induction of *rec*A gene following desiccation also established that the desiccation protocol followed was proper to and adequate to cause stress condition in *Deinococcus radiodurans*.

3.5.11. RDRM regulates radiation responsive *ssb* gene expression.

It was previously reported that RDRM (radiation/ desiccation response motif) comprising of 17 bp palindromic DNA sequences act as regulatory *cis* element preceding radiation responsive genes in at least 3 Deinococcal species including *D. radiodurans* [60, 164]. For *ssb* gene the identified RDRM (TTATGTCATTGACATAA) was located at base pairs -114 to -98, upstream of *ssb* gene open reading frame [60]. Critical examination of upstream region of *ssb* gene revealed one more somewhat similar sequence (RDRM2) (AACCGCCATCGCCAGCA) located at base pairs -213 to -197, upstream of *ssb* gene open reading frame (Fig. 3.47A). RDRM2 has only 8 bases in common to the RDRM1 at 5^{th}, 7^{th}, 8^{th}, 9^{th}, 11^{th}, 13^{th}, 14^{th} and 17^{th} position. The ΔG values for RDRM1 and RDRM2 was predicted using BPROM software at 32°C, the optimal growth temperature for *Deinococcus radiodurans*. RDRM2 has much higher GC content (64.7%) than RDRM1 (23.5%) and superior ΔG values (-32.4 kcal) versus RDRM1(-22.5 kcal). The role of these *cis* elements was assessed by constructing transcriptional fusion of these elements with a nonspecific acid

phosphatase reporter gene, *phoN*, as illustrated in Fig 3.47B. The construct pSN4 had both RDRM elements, pSN3 had only RDRM1 while pSN2 had no complete RDRM element (only the last 5 bases of the RDRM1 sequence). The differences in reporter gene enzyme activity among various constructs were visualized by zymograms. In-gel enzyme activity of PhoN in construct pSN4 showed strong induction of the reporter gene after exposure of Deinococcal cells to γ-irradiation or mitomycin C but not upon exposure to UV or hydrogen peroxide stress (Fig. 3.47C). The induction profile of various Pssb-phoN constructs was assessed further after γ-irradiation /mitomycin C treatment (Fig. 3.47 D and E). For both the stressor, the maximum in-gel enzyme activity was observed in construct pSN4, followed by construct pSN3, while only basal-level activity was seen in construct pSN2 (Fig. 3.47 D and E). Recombinant *D. radiodurans* carrying these constructs clearly revealed involvement of both RDRM sequences in activation of Ssb expression in an additive manner.

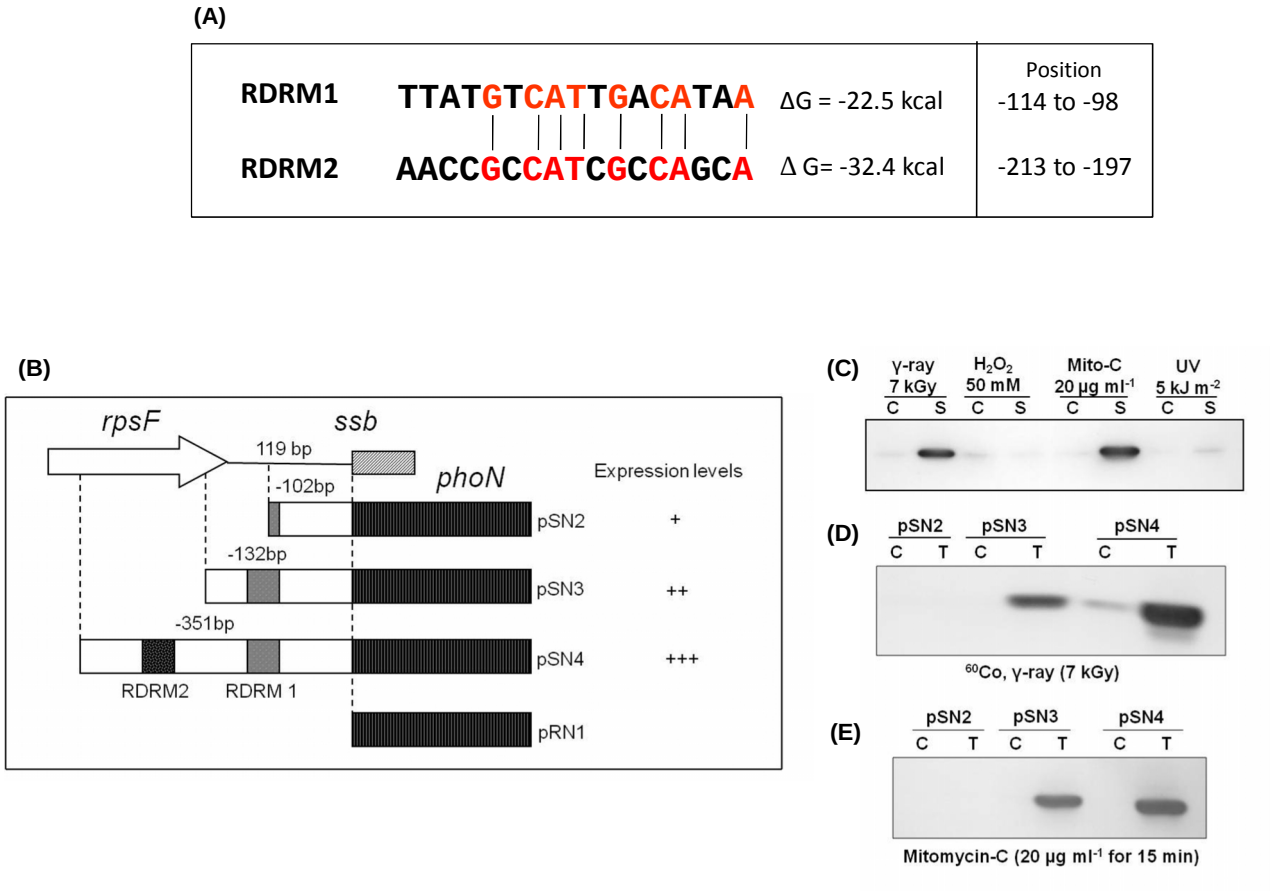


Fig. 3.47. Schematic representation of RDRM sequences and various RDRM:*pho*N constructs and their zymogram analysis. (A) Nucleotide comparison of two RDRM sequences. Conserved bases are marked in red. The loaction of two RDRM, upstream to *ssb* gene open reading frame and their ΔG values are shown. (B) Schematic representation of various *Pssb-phoN* transcriptional fusions studied and is organized with respect to *ssb* gene open reading frame. The difference in expression levels are shown by "+" sign. (C) The recombinant *D. radiodurans* strain carrying the construct pSN4 was exposed to different stresses and allowed to recover in fresh TGY medium for 3 h under the usual growth conditions. Proteins were extracted in non reducing buffer and electrophoretically resolved by 12% SDS-PAGE. Activity bands were developed by incubating the gel with NBT-BCIP in acetate buffer. (D and E), Recombinant *D. radiodurans* strains carrying either pSN2, pSN3, or pSN4 were exposed to 7 kGy of ^{60}Co gamma rays or 20 µg/ml of mitomycin C for 15 min and allowed to recover in fresh TGY medium for 3 h under the usual growth conditions.. Proteins were extracted and processed as described in panel C.

3.6. Discussion

The Ssb protein is required in various DNA related processes, such as replication, repair or recombination. This important house keeping gene keeps DNA in single stranded form by binding to it in a sequence independent manner and protecting it from nuclease or chemical degradation as well as providing these DNA intermediates as substrates for repair, replication or recombination enzymes [115, 116]. The Ssb binding to ssDNA occurs through its OB folds, using a combination of electrostatic and base stacking interaction with phosphodiester backbone and nucleotide bases of ssDNA substrate [8, 20]. The C-terminal acidic tail of Ssb protein also serves as a recruiting and docking site for various enzymes, so that they can act on the bound ssDNA. Ssb also alters the topology of DNA, so that repair proteins have better access to the substrate DNA, which in turn stimulates their activity [5].

Deinococcus-Thermus group of bacteria possess a unique Ssb in having two OB folds in comparison to one present in other bacteria [14]. The two OB folds are present distinctly at N and C-terminal region of protein with a 10 amino acids connector region connecting the two OB folds. The C-terminal OB fold resembles more with the most studied *E. coli* Ssb protein. The active Deinococcal Ssb protein is a dimer in comparison to usual tertrameric configuration observed in other eubacteria. Crystal structure of the protein has predicted that a structural asymmetry exists between the two domains and they may have evolved differently for some specialized function. The structure also showed that C-terminal region is involved in DNA binding while N-terminal domain together with the connector region is likely to be involved in multimerization [14, 19]. The occluded binding site for Deinococcal Ssb was shown to be 50±2 nucleotides and has limited capacity to displace

shorter strand in double stranded DNA molecule [18]. The major shift seen in binding mode of *E. coli* Ssb following change in ionic concentration was not seen in Deinococcal Ssb [17]. The resistance to radiation and UV is diminished if low levels of Ssb are maintained in cells. The other Ssb like Deinococcal protein-DdrB cannot complement Ssb in its function, while DrSsb can complement *E. coli ssb* gene deletion [16, 161].

The main objective of this study was to assess the importance of individual N and C-terminal domains in the overall activity of Deinococcal Ssb protein. Since the amino acid composition of both the domains was markedly different, it was expected that the two OB folds may show different biochemical activity. Three variants (N-terminal, N-terminal with connector and C-terminal) were cloned, over expressed, purified (Fig. 3.3-3.6) and compared with the full length Ssb protein for several simple and complex biochemical activities (Fig. 3.12-3.32). The connector region was cloned with N-terminal as crystal structure data had shown its role in dimerisation of Ssb protein along with N-terminal region.

The observed oligomerization pattern was different from that reported by previous studies. Full length Ssb and C-terminal formed distinct dimers in solution, while the N-terminal region existed in polydispersive forms with distinctly visible dimeric and complex multimeric forms (Fig. 3.11). The multimerization property of N-terminal domain may provide high stability to dimeric behaviour of Deinococcal Ssb protein with C-terminal region also showing dimer formation tendency. EMSA studies with dT50 oligos clearly demonstrated the differential ssDNA binding activity of individual domains. The full length Ssb showed maximum affinity for ssDNA followed by C-terminal domain. The co-operativity of Ssb_{FL} was also higher than its C-terminal counterpart. N-terminal with

connector showed a smear like pattern of binding to ssDNA with a distinct decrease in target DNA. Such pattern was previously linked to cooperativity by others [17, 177, 178]. The N-terminal region alone showed a very different pattern in comparison to its counterpart with connector. Few discrete DNA-protein complex bands were seen with very little decrease in target DNA. This differential behavior in N-terminal binding activity of Ssb_N and Ssb_{NC} shows the influence of connector region in affecting binding pattern to the target DNA (Fig. 3.12). The combination EMSA with N and C-terminal domains showed that the interaction of N and C-terminal domains leads to generation of a functional complex which has a higher affinity for ssDNA, in comparison to individual domains. The connector region also played a role in combination assay with combination of Ssb_{NC}/Ssb_C showing a different binding mode than that of Ssb_N/Ssb_C (Fig. 3.13). It is clear that N-terminal region along with connector region is actively involved in ssDNA binding in combination with Ssb_C. Further, the influence of connector region of Ssb_{NC} in forming a different functional complex with C-terminal region cannot be ruled out.

Ssb protein is also involved in altering the topology of DNA, thereby enhancing the activity of many repair proteins including topoisomerase I and RecA [5]. In topoisomearse assay it stabilizes the single stranded region at the topoisomerase site, which acts as a sink for topoisomerase I thus enhancing its activity. All Ssb variants were able to differentially modulate topoisomerase I activity of *E. coli*. In presence of Ssb_{FL}, topoisomerase I showed rapid processive behavior, forming a single band of relaxed DNA (Fig. 3.18). In presence of other Ssb variants or their combinations, topoisomerase I showed a distributive behavior, leading to formation of different intermediate topoisomers (Fig. 3.19). This difference in activity may be attributed to different affinity for single stranded DNA at topoisomerase I

action site with different Ssb variants, as well as accessibility of Ssb variants to such site depending on the solution status of various variants or their combination. The inhibitory effect in topoisomerase I activity at higher concentration of Ssb variants, especially in combination, (Fig. 3.19) may be due to competition for single stranded DNA with Ssb and topoisomerase I.

Unlike the full length Ssb, all Ssb variants or their combination were not able to remove secondary structure of ssDNA used as substrate in strand exchange with cognate Deinococcal RecA or noncognate *E. coli* RecA (Fig. 3.23-3.30). Since EMSA conducted in strand exchange assay condition confirmed binding by all variants to ssDNA (Fig. 3.31), the non-formation of nicked circular product molecule can be definitely linked to non-removal of secondary structure of ssDNA substrate, a process which Ssb_{FL} easily does as it contains naturally linked N and C-terminal domains.

Based on the biochemical data a model is proposed which is shown in Fig. 3.48.

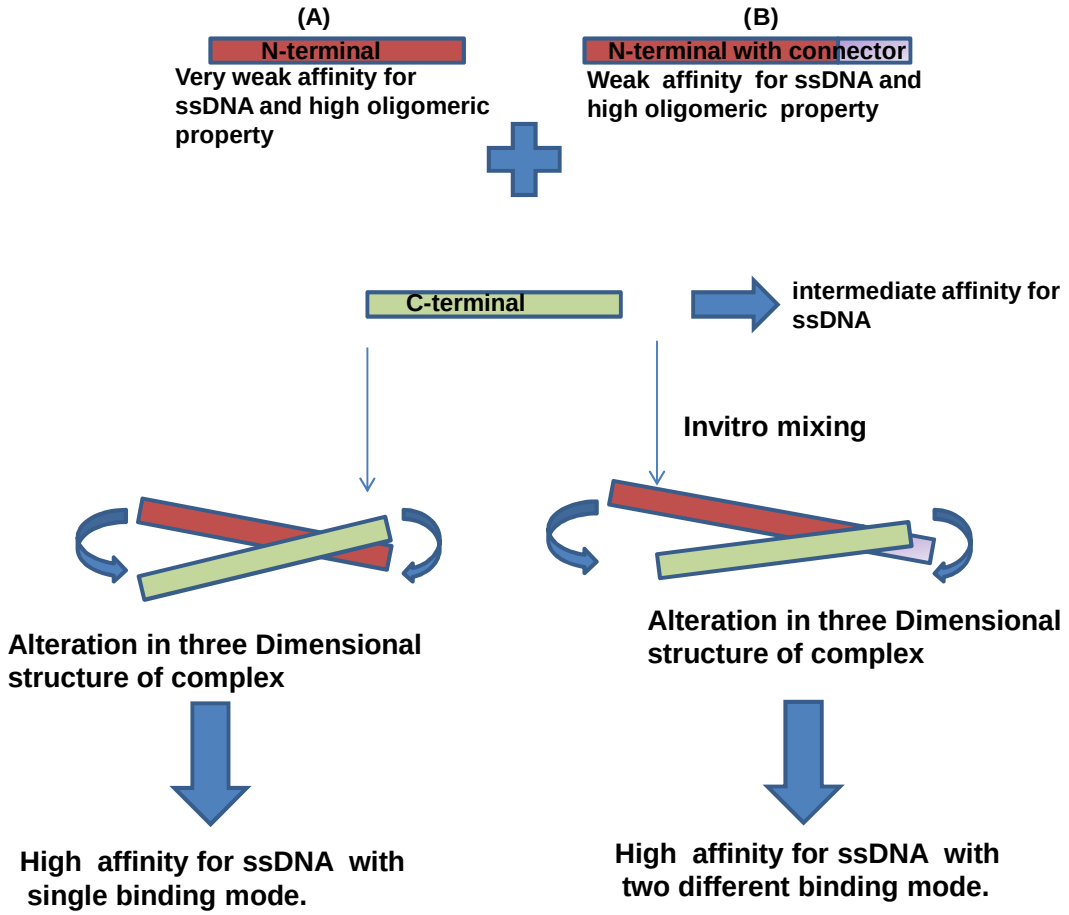


Fig. 3.48. Model for *in vitro* interaction of Ssb N-terminal and C-terminal regions. $Ssb_{N,}$ Ssb_{NC} and Ssb_{C}, *per se* show differential binding to ssDNA. *In vitro* interaction of the (A) Ssb_{N}/Ssb_{C} and (B) Ssb_{NC}/Ssb_{C} leads to different alteration in three dimensional complex formed individually, which results in high affinity for ssDNA with different binding mode.

The real life situation is similar to the scheme shown in (B) than in (A). However, the *in vitro* binding pattern of Ssb_{FL} is more similar to the combination of Ssb_{N}/Ssb_{C} shown in (A). The saturation in binding at equimolar concentrations in combination assays, showed that Ssb_{NC}/Ssb_{C} combination has a higher affinity for ssDNA in comparison to Ssb_{N}/Ssb_{C} combination (Fig. 3.13). Two binding modes

displayed by Ssb_{NC}/Ssb_{C} combination (Fig. 3.13) differ from the single binding mode shown by Ssb_{FL} (Fig. 3.12) and may merely reflect that connector region modifies the interaction between N and C terminal OB folds. The interaction of N-terminal region is specific for its cognate C-terminal partner only as no increase in *E. coli* Ssb binding activity was observed when titrated with increasing concentration of N-terminal (Fig. 3.14).

The massive DNA strand breaks caused by radiation/mitomycin-C etc. resulted in significant induction of Ssb levels in *D. radiodurans* (Fig. 3.34-3.39). The non-induction of Ssb levels at high dose rate of gamma radiation is somewhat inexplicable, but may be because radiation is a 'fleeting stress' and causes less damage at high dose rate due to less interaction with DNA compared to that at lower dose rate. This is supported by much higher D_{10} survival values of *Deinococcus radiodurans* at high dose rate (19.2 kGy) than at low dose rate (10.2 kGy). The lag observed following irradiation is also higher in case of low dose irradiation (4 h) than after high dose irradiation (2 h) [179]. Surprisingly desiccation stress of 6 weeks also did not enhance Ssb levels. It is well known that the DNA damage caused by 3 to 5 kGy of gamma irradiation [30, 91] is much more than that caused by four weeks of desiccation. It is likely that a high threshold level of DSBs is required for induction of *ssb* expression to occur. Acute irradiation can easily exceed this threshold, while chronic slow-acting desiccation stress may not be able to cross this limit.

Ssb is known to play a very important role in protecting damaged DNA from nucleolytic cleavage as well as providing the bound ssDNA:Ssb intermediates as substrates for various repair mechanism. Strong induction of Ssb following extensive

DSBs caused by γ-rays and mitomycin-C is a clear indication that Deinococcal Ssb is available in high levels to play such a role in post-stress DNA protection and repair. The *ssb* gene harbours two RDRM sequences in its promoter region. This conserved 17 base pair palindromic sequence has been proposed to act as a *cis* regulatory elements and together with global regulator DdrO is thought to regulate expression of DNA repair genes [60], though an experimental evidence was so far lacking. Both RDRM sequences were found to contribute additively in enhancement of *ssb* gene expression as assessed by the reporter gene analysis. RDRM 2 had better ΔG (-32.4 kcal, versus -22.5 kcal for RDRM1) values and is conserved at the same location in *ssb* gene of at least 3 species of *Deinococcus*. Whether the RDRM sequences are adequate in controlling *ssb* gene expression pre and post DNA damage stress or need a transacting protein remains to be established. As no enhancement of Ssb levels was seen in case of 6 weeks desiccation stress, it has been proposed to rename this regulatory element to RRM (radiation response motif) [164].

To conclude, both N and C-terminal domain of Ssb showed differential ssDNA binding activity. The combination of both N and C-terminal OB folds *in trans* contributed additively in DNA binding. N-terminal domain may further stabilize the dimer formation of C-terminal domain due to its multimerization property. *In trans* contribution of individual OB fold is sufficient to stimulate activity of topoisomerase I, while cooperative contribution of both OB folds in the naturally occurring *cis* state of Ssb_{FL} is required for stimulating more complex activity of strand exchange promoted by RecA.

Some unanswered questions do remain. Why the structural asymmetry because of differences in amino acid composition of two OB folds has evolved in *Deinococcus-Thermus* group of bacteria, since biochemical data resemble *E. coli* Ssb with only few minor differences. The role of Ssb appears to be shared among the two OB folds in Deinococcal Ssb. The question remains if Deinococcal Ssb has evolved for any un explored repair process or it's mere coincidence that two OB folds were duplicated and acquired changes differently in the course of evolution. Role of RDRM in radiation responsive expression of many more characterized and un characterized ORFs of *Deinococcus* is necessary to elucidate underlying mechanistic details. Similarly role of N and C terminal domain interaction in Ssb in recruiting and activity of other interacting partners of Ssb protein deserves further exploration.

Chapter 4
Summary and Conclusions

Deinococcus radiodurans is known to survive in extreme environments, including exposure to acute and extremely high doses of ionizing radiation and prolonged desiccation, that cause massive breach in cellular and DNA integrity by direct and indirect effects. These effects include double strand breaks, single strand breaks, individual base damage and oxidation of proteins, lipids and other biomolecules. The survival strategy of *Deinococcus radiodurans* against such damage is multifaceted and involves (i) condensed nucleiod structure, (ii) robust DNA repair mechanism involving ESDSA (Extended synthesis dependant strand annealing) pathway, (iii) adaptation of energy metabolism to reduce generation of reactive oxygen species, (iv) high Mn/Fe ratio and soluble Mn^{2+} complexes of biomolecules, which act as potent ROS scavengers, and (v) robust ABC transporter system for quick processing of damaged nucleotides and proteins.

Single stranded DNA binding protein (Ssb) is required in all aspects of DNA metabolism such as replication, recombination or repair. Expectedly, Ssb levels are significantly elevated in *Deinococcus* during post irradiation recovery (PIR), as revealed both by transcriptomic as well as proteomic data. The underlying mechanism are, however, largely unknown. Deinococcal genome harbors conserved palindromic DNA sequences designated as RDRM (Radiation and Desiccation Response Motif). The sequence generally precedes genes, which are induced following radiation and desiccation stress and is conserved across at least 3 different Deinococcal species. Recently these regulatory elements are thought to be controlled by global regulator DdrO in *Deinococcus* lineages. Two such RDRM sequences are also present upstream of the

ssb gene. The possibility that they may play a role in radiation responsive Ssb expression has remained unexplored so far.

Typically the interaction of Ssb with DNA occurs through a structurally conserved OB (Oligonucleotide/ oligosaccharide binding) fold domain. Interaction with DNA repair proteins occurs through its C-terminal acidic tail. Ssb proteins also have oligomerization property which leads to differences in number of OB folds interacting with DNA across different organisms. The most studied eubacterial Ssb from *E. coli* has a single OB fold and it exists and functions predominantly as a tetramer. Ssb modulates the activity of repair, recombination and replication proteins either by direct interaction with proteins through its C-terminal acidic tail or indirectly by altering the topology of DNA so that repair proteins can interact optimally with DNA.

Deinococcus radiodurans Ssb protein comprises of 2 asymmetric OB folds linked by a 10 amino acid long connector. The evolution of a unique Ssb protein with twin OB folds in *Deinococcus-Thermus* group of bacteria suggests a likely specialized function for each domain. Bioinformatic analyses together with crystal structure data on Deinococcal Ssb have earlier implicated C-terminal OB fold in ssDNA binding and the N-terminal OB fold and connector region in dimer formation. To evaluate these possibilities, different truncated Ssb variants were constructed in the present study and their interactions studied. Availability of enhanced Ssb levels during post DNA damage was also examined and DNA damage induced regulation was investigated to reveal underlying mechanism. The data presented in this thesis have revealed following new information regarding structure-based functional significance of individual OB folds in the novel Deinococcal

Ssb protein and partly elucidated the molecular basis of upregulation of Ssb in response to intense DNA damage:

1. The ssDNA binding capability of *D. radiodurans* Ssb primarily resides in the C-terminal OB fold while the N-terminal OB fold is engaged in oligomerization. The two cognate OB folds interact physically and functionally, to display improved ssDNA binding *in vitro*. Since major proportion of N-terminal region *per se* largely existed as a complex multimer, possibility of its misfolding in the absence of C-terminal region cannot be ruled out. But ssDNA binding by Ssb_N/Ssb_{NC} and enhanced DNA binding upon interaction of N-terminal region with C-terminal region indicates correct folding and conformation of the DNA-protein complex. It is therefore proposed that the N-terminal region serves two types of functions: (1) it facilitates oligomerization, and (2) interacts with C-terminal region *in vitro* to enhance the affinity of the complex for ssDNA, over and above that of individual N and C-terminal OB folds. The interaction of N-terminal OB folds with its cognate C-terminal partner is highly specific with potential functional implications. A similar functional interaction was not observed with non-cognate *E. coli* Ssb protein.
2. The individual N and C terminal domains or their *in trans* combination were able to stimulate *E. coli* topoisomerase I activity to display a distributive pattern and generation of intermediate DNA topoisomers. In contrast, the full length native Ssb_{FL}, supported a rapid processive activity of topoisomerase I, leading to generation of fully relaxed DNA molecule(s). The observed differences in the modulation of topoisomerase I activity shown by different Ssb variants may be attributed to differences in their ability to mediate melting of DNA at topoisomerase I reaction site as well as accessibility to such sites,

possibly depending on their oligomeric status. Combination of Ssb_N/Ssb_{NC} with Ssb_C did not result in processive behavior of topoisomerase I, like shown by Ssb_{FL}.

3. Unlike Ssb_{FL}, the Ssb_N, Ssb_{NC} or Ssb_C variants or their combinations could not effect the more complex strand exchange activity by cognate Deinococcal RecA either in SS-DS or DS-SS mode or by the non-cognate *E. coli* RecA in SS-DS mode. Inefficient removal of the secondary structures of ssDNA substrates by different Ssb variants (or their combinations), along with their differential affinity for ssDNA may account for non-stimulation of RecA mediated strand exchange. Ssb_{FL}, which contains naturally linked N and C-terminal domains, probably has the flexibility of interacting the two domains in *cis* state to remove the secondary structure of ssDNA substrate much more efficiently, thereby enhancing RecA activity.
4. The extent and nature of damage to DNA integrity determines the upregulation in cellular levels of Ssb protein. Gamma irradiation and mitomycin-C cause maximum breach in DNA integrity leading to extensive double strand breaks, single strand breaks and other base damages and significantly enhance expression of Ssb during post-stress recovery. In comparison UV, hydrogen peroxide or six weeks of desiccation result in relatively mild DNA damage which may be taken care by Ssb level already present and did not cause fresh induction of *ssb* expression. The DNA damage responsive expression of *ssb* gene is transcriptionally induced, and indeed controlled by the two radiation and desiccation response motifs (RDRM) present upstream of *ssb* gene, which seem to work additively. The enhanced Ssb expression in turn plays the important role of keeping DNA in single stranded conformation for repair, recombination and simultaneously protecting it from nuclease digestion during PIR.

The evolution of the atypical Deinococcal Ssb appears to be prompted by a necessity to protect as much ssDNA templates as possible, following stress-induced massive DNA damage. Specific interactions and cooperative contribution of both the OB folds is necessary for efficient melting of secondary structures in ssDNA, a feature essential for DNA replication, recombination and repair. Cooperative interactions between multimerization inducing N-terminal OB fold and ssDNA binding C-terminal OB fold appear to functionally complement each other in ssDNA binding to accomplish rapid nucleation and, thereby, protection of ssDNA templates to be used for very efficient and error-free post-damage DNA repair in this superbug.

References

1. de Groot, A., Chapon, V., Servant, P., Christen, R., Saux, M. F., Sommer, S. & Heulin, T. (2005) *Deinococcus deserti* sp. nov., a gamma-radiation-tolerant bacterium isolated from the Sahara Desert, *International journal of systematic and evolutionary microbiology.* **55**, 2441-6.

2. Zahradka, K., Slade, D., Bailone, A., Sommer, S., Averbeck, D., Petranovic, M., Lindner, A. B. & Radman, M. (2006) Reassembly of shattered chromosomes in *Deinococcus radiodurans*, *Nature.* **443**, 569-73.

3. Daly, M. J., Gaidamakova, E. K., Matrosova, V. Y., Vasilenko, A., Zhai, M., Venkateswaran, A., Hess, M., Omelchenko, M. V., Kostandarithes, H. M., Makarova, K. S., Wackett, L. P., Fredrickson, J. K. & Ghosal, D. (2004) Accumulation of Mn(II) in *Deinococcus radiodurans* facilitates gamma-radiation resistance, *Science.* **306**, 1025-8.

4. Levin-Zaidman, S., Englander, J., Shimoni, E., Sharma, A. K., Minton, K. W. & Minsky, A. (2003) Ringlike structure of the *Deinococcus radiodurans* genome: a key to radioresistance?, *Science.* **299**, 254-6.

5. Shereda, R. D., Kozlov, A. G., Lohman, T. M., Cox, M. M. & Keck, J. L. (2008) SSB as an organizer/mobilizer of genome maintenance complexes, *Crit Rev Biochem Mol Biol.* **43**, 289-318.

6. Casas-Finet, J. R., Khamis, M. I., Maki, A. H. & Chase, J. W. (1987) Tryptophan 54 and phenylalanine 60 are involved synergistically in the binding of *E. coli* SSB protein to single-stranded polynucleotides, *FEBS Lett.* **220**, 347-52.

7. George, N. P., Ngo, K. V., Chitteni-Pattu, S., Norais, C. A., Battista, J. R., Cox, M. M. & Keck, J. L. (2012) Structure and cellular dynamics of *Deinococcus radiodurans* single-

stranded DNA (ssDNA)-binding protein (SSB)-DNA complexes, *J Biol Chem.* **287**, 22123-32.

8. Overman, L. B. & Lohman, T. M. (1994) Linkage of pH, anion and cation effects in protein-nucleic acid equilibria. *Escherichia coli* SSB protein-single stranded nucleic acid interactions, *J Mol Biol.* **236**, 165-78.

9. Slade, D. & Radman, M. (2011) Oxidative stress resistance in *Deinococcus radiodurans*, *Microbiol Mol Biol Rev.* **75**, 133-91.

10. Meyer, R. R. & Laine, P. S. (1990) The single-stranded DNA-binding protein of *Escherichia coli*, *Microbiol Rev.* **54**, 342-80.

11. Furukohri, A., Nishikawa, Y., Akiyama, M. T. & Maki, H. (2012) Interaction between *Escherichia coli* DNA polymerase IV and single-stranded DNA-binding protein is required for DNA synthesis on SSB-coated DNA, *Nucleic Acids Res.* **40**, 6039-48.

12. Reddy, M. S., Guhan, N. & Muniyappa, K. (2001) Characterization of single-stranded DNA-binding proteins from Mycobacteria. The carboxyl-terminal of domain of SSB is essential for stable association with its cognate RecA protein, *J Biol Chem.* **276**, 45959-68.

13. Antony, E., Weiland, E., Yuan, Q., Manhart, C. M., Nguyen, B., Kozlov, A. G., McHenry, C. S. & Lohman, T. M. (2013) Multiple C-terminal tails within a single *E. coli* SSB homotetramer coordinate DNA replication and repair, *J Mol Biol.* **425**, 4802-19.

14. Eggington, J. M., Haruta, N., Wood, E. A. & Cox, M. M. (2004) The single-stranded DNA-binding protein of *Deinococcus radiodurans*, *BMC Microbiol.* **4**, 2.

15. Wold, M. S. (1997) Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism, *Annu Rev Biochem.* **66**, 61-92.

16. Lockhart, J. S. & DeVeaux, L. C. (2013) The essential role of the *Deinococcus radiodurans* ssb gene in cell survival and radiation tolerance, *PLoS One.* **8**, e71651.

17. Kozlov, A. G., Eggington, J. M., Cox, M. M. & Lohman, T. M. (2010) Binding of the dimeric *Deinococcus radiodurans* single-stranded DNA binding protein to single-stranded DNA, *Biochemistry.* **49**, 8266-75.

18. Eggington, J. M., Kozlov, A. G., Cox, M. M. & Lohman, T. M. (2006) Polar destabilization of DNA duplexes with single-stranded overhangs by the *Deinococcus radiodurans* SSB protein, *Biochemistry.* **45**, 14490-502.

19. Bernstein, D. A., Eggington, J. M., Killoran, M. P., Misic, A. M., Cox, M. M. & Keck, J. L. (2004) Crystal structure of the *Deinococcus radiodurans* single-stranded DNA-binding protein suggests a mechanism for coping with DNA damage, *Proc Natl Acad Sci U S A.* **101**, 8575-80.

20. Raghunathan, S., Kozlov, A. G., Lohman, T. M. & Waksman, G. (2000) Structure of the DNA binding domain of *E. coli* SSB bound to ssDNA, *Nat Struct Biol.* **7**, 648-52.

21. Muniyappa, K., Shaner, S. L., Tsang, S. S. & Radding, C. M. (1984) Mechanism of the concerted action of recA protein and helix-destabilizing proteins in homologous recombination, *Proc Natl Acad Sci U S A.* **81**, 2757-61.

22. Kowalczykowski, S. C. & Krupp, R. A. (1987) Effects of *Escherichia coli* SSB protein on the single-stranded DNA-dependent ATPase activity of *Escherichia coli* RecA protein. Evidence that SSB protein facilitates the binding of RecA protein to regions of secondary structure within single-stranded DNA, *J Mol Biol.* **193**, 97-113.

23. Ngo, K. V., Molzberger, E. T., Chitteni-Pattu, S. & Cox, M. M. (2013) Regulation of *Deinococcus radiodurans* RecA protein function via modulation of active and inactive nucleoprotein filament states, *J Biol Chem.* **288**, 21351-66.

24. Jain, S., Zweig, M., Peeters, E., Siewering, K., Hackett, K. T., Dillard, J. P. & van der Does, C. (2012) Characterization of the single stranded DNA binding protein SsbB encoded in the Gonoccocal Genetic Island, *PLoS One.* **7**, e35285.

25. Sikder, D., Unniraman, S., Bhaduri, T. & Nagaraja, V. (2001) Functional cooperation between topoisomerase I and single strand DNA-binding protein, *J Mol Biol.* **306**, 669-79.

26. Murray, R. G. (1986) Family II. *Deinococcaceae*,

, *Bergey's manual of systematic bacteriology Williams & Wilkins Co, Baltimore, MD*

2, 1035–1043.

27. He, Y. (2009) High cell density production of *Deinococcus radiodurans* under optimized conditions, *Journal of industrial microbiology & biotechnology.* **36**, 539-46.

28. Brim, H., Venkateswaran, A., Kostandarithes, H. M., Fredrickson, J. K. & Daly, M. J. (2003) Engineering *Deinococcus geothermalis* for bioremediation of high-temperature radioactive waste environments, *Applied and environmental microbiology.* **69**, 4575-82.

29. Appukuttan, D., Rao, A. S. & Apte, S. K. (2006) Engineering of *Deinococcus radiodurans* R1 for bioprecipitation of uranium from dilute nuclear waste, *Applied and environmental microbiology.* **72**, 7873-8.

30. Battista, J. R. (1997) Against all odds: the survival strategies of *Deinococcus radiodurans*, *Annual review of microbiology.* **51**, 203-24.

31. Sanders, S. W. & Maxcy, R. B. (1979) Isolation of radiation-resistant bacteria without exposure to irradiation, *Applied and environmental microbiology.* **38**, 436-9.

32. Blasius, M., Sommer, S. & Hubscher, U. (2008) *Deinococcus radiodurans*: what belongs to the survival kit?, *Crit Rev Biochem Mol Biol.* **43**, 221-38.

33. Anderson, A. W., H. C. Nordan, R. F. Cain, G. Parrish, and D. Duggan. (1956) Studies on a radio-resistant *micrococcous*. I. Isolation, morphology, cultural characteristics, and resistance to gamma radiation., *Food Technology.* **10**, 575-577.

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

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

36. Omelchenko, M. V., Wolf, Y. I., Gaidamakova, E. K., Matrosova, V. Y., Vasilenko, A., Zhai, M., Daly, M. J., Koonin, E. V. & Makarova, K. S. (2005) Comparative genomics of *Thermus thermophilus* and *Deinococcus radiodurans*: divergent routes of adaptation to thermophily and radiation resistance, *BMC evolutionary biology.* **5**, 57.

37. Albuquerque, L., Simoes, C., Nobre, M. F., Pino, N. M., Battista, J. R., Silva, M. T., Rainey, F. A. & da Costa, M. S. (2005) *Truepera radiovictrix* gen. nov., sp. nov., a new radiation resistant species and the proposal of *Trueperaceae* fam. nov, *FEMS microbiology letters.* **247**, 161-9.

38. Work, E. & Griffiths, H. (1968) Morphology and chemistry of cell walls of *Micrococcus radiodurans*, *J Bacteriol.* **95**, 641-57.

39. Lange, C. C., Wackett, L. P., Minton, K. W. & Daly, M. J. (1998) Engineering a recombinant *Deinococcus radiodurans* for organopollutant degradation in radioactive mixed waste environments, *Nature biotechnology.* **16**, 929-33.

40. Anderson, R. & Hansen, K. (1985) Structure of a novel phosphoglycolipid from *Deinococcus radiodurans*, *J Biol Chem.* **260**, 12219-23.

41. Knivett, V. A., Cullen, J. & Jackson, M. J. (1965) Odd-Numbered Fatty Acids in *Micrococcus Radiodurans*, *The Biochemical journal.* **96**, 2C-3C.

42. Girard, A. E. (1971) A comparative study of the fatty acids of some micrococci, *Canadian journal of microbiology.* **17**, 1503-8.

43. Murray, R. G., Hall, M. & Thompson, B. G. (1983) Cell division in *Deinococcus radiodurans* and a method for displaying septa, *Canadian journal of microbiology.* **29**, 1412-23.

44. Daly, M. J., Gaidamakova, E. K., Matrosova, V. Y., Vasilenko, A., Zhai, M., Leapman, R. D., Lai, B., Ravel, B., Li, S. M., Kemner, K. M. & Fredrickson, J. K. (2007) Protein oxidation implicated as the primary determinant of bacterial radioresistance, *PLoS biology.* **5**, e92.

45. Zhang, Y. M., Wong, T. Y., Chen, L. Y., Lin, C. S. & Liu, J. K. (2000) Induction of a futile Embden-Meyerhof-Parnas pathway in *Deinococcus radiodurans* by Mn: possible role of the pentose phosphate pathway in cell survival, *Applied and environmental microbiology.* **66**, 105-12.

46. Ghosal, D., Omelchenko, M. V., Gaidamakova, E. K., Matrosova, V. Y., Vasilenko, A., Venkateswaran, A., Zhai, M., Kostandarithes, H. M., Brim, H., Makarova, K. S., Wackett, L. P., Fredrickson, J. K. & Daly, M. J. (2005) How radiation kills cells: survival of

Deinococcus radiodurans and Shewanella oneidensis under oxidative stress, *FEMS microbiology reviews.* **29**, 361-75.

47. Liu, Y., Zhou, J., Omelchenko, M. V., Beliaev, A. S., Venkateswaran, A., Stair, J., Wu, L., Thompson, D. K., Xu, D., Rogozin, I. B., Gaidamakova, E. K., Zhai, M., Makarova, K. S., Koonin, E. V. & Daly, M. J. (2003) Transcriptome dynamics of *Deinococcus radiodurans* recovering from ionizing radiation, *Proc Natl Acad Sci U S A.* **100**, 4191-6.

48. Daly, M. J., Gaidamakova, E. K., Matrosova, V. Y., Kiang, J. G., Fukumoto, R., Lee, D. Y., Wehr, N. B., Viteri, G. A., Berlett, B. S. & Levine, R. L. (2010) Small-molecule antioxidant proteome-shields in *Deinococcus radiodurans*, *PLoS One.* **5**, e12570.

49. Halliwell, B., and J. M. Gutteridge. (1999) Free radicals in biology and medicine,, *Oxford University Press, Oxford, United Kingdom.* **3**.

50. Zhang, C., Wei, J., Zheng, Z., Ying, N., Sheng, D. & Hua, Y. (2005) Proteomic analysis of *Deinococcus radiodurans* recovering from gamma-irradiation, *Proteomics.* **5**, 138-43.

51. Imlay, J. A. (2003) Pathways of oxidative damage, *Annual review of microbiology.* **57**, 395-418.

52. Kornberg, A. (1995) Inorganic polyphosphate: toward making a forgotten polymer unforgettable, *J Bacteriol.* **177**, 491-6.

53. Rao, N. N., Gomez-Garcia, M. R. & Kornberg, A. (2009) Inorganic polyphosphate: essential for growth and survival, *Annu Rev Biochem.* **78**, 605-47.

54. Wood, H. G. & Clark, J. E. (1988) Biological aspects of inorganic polyphosphates, *Annu Rev Biochem.* **57**, 235-60.

55. Moseley, B. E. & Schein, A. H. (1964) Radiation Resistance and Deoxyribonucleic Acid Base Composition of *Micrococcus Radiodurans*, *Nature.* **203**, 1298-9.

56. White, O., Eisen, J. A., Heidelberg, J. F., Hickey, E. K., Peterson, J. D., Dodson, R. J., Haft, D. H., Gwinn, M. L., Nelson, W. C., Richardson, D. L., Moffat, K. S., Qin, H., Jiang, L., Pamphile, W., Crosby, M., Shen, M., Vamathevan, J. J., Lam, P., McDonald, L., Utterback, T., Zalewski, C., Makarova, K. S., Aravind, L., Daly, M. J., Minton, K. W., Fleischmann, R. D., Ketchum, K. A., Nelson, K. E., Salzberg, S., Smith, H. O., Venter, J. C. & Fraser, C. M. (1999) Genome sequence of the radioresistant bacterium *Deinococcus radiodurans* R1, *Science.* **286**, 1571-7.

57. Harsojo, Kitayama, S. & Matsuyama, A. (1981) Genome multiplicity and radiation resistance in *Micrococcus radiodurans*, *Journal of biochemistry.* **90**, 877-80.

58. Hansen, M. T. (1978) Multiplicity of genome equivalents in the radiation-resistant bacterium *Micrococcus radiodurans*, *J Bacteriol.* **134**, 71-5.

59. Moseley, B. E. & Copland, H. J. (1975) Isolation and properties of a recombination-deficient mutant of *Micrococcus radiodurans*, *J Bacteriol.* **121**, 422-8.

60. Makarova, K. S., Omelchenko, M. V., Gaidamakova, E. K., Matrosova, V. Y., Vasilenko, A., Zhai, M., Lapidus, A., Copeland, A., Kim, E., Land, M., Mavrommatis, K., Pitluck, S., Richardson, P. M., Detter, C., Brettin, T., Saunders, E., Lai, B., Ravel, B., Kemner, K. M., Wolf, Y. I., Sorokin, A., Gerasimova, A. V., Gelfand, M. S., Fredrickson, J. K., Koonin, E. V. & Daly, M. J. (2007) *Deinococcus geothermalis*: the pool of extreme radiation resistance genes shrinks, *PLoS One.* **2**, e955.

61. Daly, M. J. (2009) A new perspective on radiation resistance based on *Deinococcus radiodurans*, *Nature reviews Microbiology.* **7**, 237-45.

62. Bjelland, S. & Seeberg, E. (2003) Mutagenicity, toxicity and repair of DNA base damage induced by oxidation, *Mutation research.* **531**, 37-80.

63. Hariharan, P. V. & Cerutti, P. A. (1972) Formation and repair of gamma-ray induced thymine damage in *Micrococcus radiodurans*, *J Mol Biol.* **66**, 65-81.

64. Burrell, A. D., Feldschreiber, P. & Dean, C. J. (1971) DNA-membrane association and the repair of double breaks in x-irradiated *Micrococcus radiodurans*, *Biochimica et biophysica acta.* **247**, 38-53.

65. Kish, A., Kirkali, G., Robinson, C., Rosenblatt, R., Jaruga, P., Dizdaroglu, M. & DiRuggiero, J. (2009) Salt shield: intracellular salts provide cellular protection against ionizing radiation in the halophilic archaeon, *Halobacterium salinarum* NRC-1, *Environmental microbiology.* **11**, 1066-78.

66. Bonura, T. & Bruce, A. K. (1974) The repair of single-strand breaks in a radiosensitive mutant of *Micrococcus radiodurans*, *Radiation research.* **57**, 260-75.

67. Dean, C. J., Little, J. G. & Serianni, R. W. (1970) The control of post irradiation DNA breakdown in *Micrococcus radiodurans*, *Biochemical and biophysical research communications.* **39**, 126-34.

68. Kitayama, S. & Matsuyama, A. (1976) DNA synthesis and repair in permeable cells of *Micrococcus radiodurans*, *Biochimica et biophysica acta.* **418**, 321-9.

69. Driedger, A. A. & Grayston, M. J. (1971) Demonstration of two types of DNA repair in X-irradiated *Micrococcus radiodurans*, *Canadian journal of microbiology.* **17**, 495-9.

70. Slade, D., Lindner, A. B., Paul, G. & Radman, M. (2009) Recombination and replication in DNA repair of heavily irradiated *Deinococcus radiodurans*, *Cell.* **136**, 1044-55.

71. Masters, C. I., Moseley, B. E. & Minton, K. W. (1991) AP endonuclease and uracil DNA glycosylase activities in *Deinococcus radiodurans*, *Mutation research.* **254**, 263-72.

72. Mennecier, S., Coste, G., Servant, P., Bailone, A. & Sommer, S. (2004) Mismatch repair ensures fidelity of replication and recombination in the radioresistant organism *Deinococcus radiodurans*, *Molecular genetics and genomics : MGG.* **272**, 460-9.

73. Nguyen, H. H., de la Tour, C. B., Toueille, M., Vannier, F., Sommer, S. & Servant, P. (2009) The essential histone-like protein HU plays a major role in *Deinococcus radiodurans* nucleoid compaction, *Molecular microbiology.* **73**, 240-52.

74. Bhattacharyya, G. & Grove, A. (2007) The N-terminal extensions of *Deinococcus radiodurans* Dps-1 mediate DNA major groove interactions as well as assembly of the dodecamer, *J Biol Chem.* **282**, 11921-30.

75. Hipkiss, A. R. (2006) Accumulation of altered proteins and ageing: causes and effects, *Experimental gerontology.* **41**, 464-73.

76. Leibowitz, P. J., Schwartzberg, L. S. & Bruce, A. K. (1976) The in vivo association of manganese with the chromosome of *Micrococcus radiodurans*, *Photochemistry and photobiology.* **23**, 45-50.

77. Daly, M. J. (2006) Modulating radiation resistance: Insights based on defenses against reactive oxygen species in the radioresistant bacterium *Deinococcus radiodurans*, *Clinics in laboratory medicine.* **26**, 491-504, x.

78. Tian, B., Wu, Y., Sheng, D., Zheng, Z., Gao, G. & Hua, Y. (2004) Chemiluminescence assay for reactive oxygen species scavenging activities and inhibition on oxidative damage of DNA in *Deinococcus radiodurans*, *Luminescence : the journal of biological and chemical luminescence.* **19**, 78-84.

79. Wang, L., Xu, G., Chen, H., Zhao, Y., Xu, N., Tian, B. & Hua, Y. (2008) DrRRA: a novel response regulator essential for the extreme radioresistance of *Deinococcus radiodurans*, *Molecular microbiology.* **67**, 1211-22.

80. Chen, H., Xu, G., Zhao, Y., Tian, B., Lu, H., Yu, X., Xu, Z., Ying, N., Hu, S. & Hua, Y. (2008) A novel OxyR sensor and regulator of hydrogen peroxide stress with one cysteine residue in *Deinococcus radiodurans*, *PLoS One.* **3**, e1602.

81. Abreu, I. A., Hearn, A., An, H., Nick, H. S., Silverman, D. N. & Cabelli, D. E. (2008) The kinetic mechanism of manganese-containing superoxide dismutase from *Deinococcus radiodurans*: a specialized enzyme for the elimination of high superoxide concentrations, *Biochemistry.* **47**, 2350-6.

82. Dennis, R. J., Micossi, E., McCarthy, J., Moe, E., Gordon, E. J., Kozielski-Stuhrmann, S., Leonard, G. A. & McSweeney, S. (2006) Structure of the manganese superoxide dismutase from *Deinococcus radiodurans* in two crystal forms, *Acta crystallographica Section F, Structural biology and crystallization communications.* **62**, 325-9.

83. Markillie, L. M., Varnum, S. M., Hradecky, P. & Wong, K. K. (1999) Targeted mutagenesis by duplication insertion in the radioresistant bacterium *Deinococcus radiodurans*: radiation sensitivities of catalase (katA) and superoxide dismutase (sodA) mutants, *J Bacteriol.* **181**, 666-9.

84. Tian, B., Xu, Z., Sun, Z., Lin, J. & Hua, Y. (2007) Evaluation of the antioxidant effects of carotenoids from *Deinococcus radiodurans* through targeted mutagenesis, chemiluminescence, and DNA damage analyses, *Biochimica et biophysica acta.* **1770**, 902-11.

85. Zhang, L., Yang, Q., Luo, X., Fang, C., Zhang, Q. & Tang, Y. (2007) Knockout of crtB or crtI gene blocks the carotenoid biosynthetic pathway in *Deinococcus radiodurans* R1 and influences its resistance to oxidative DNA-damaging agents due to change of free radicals scavenging ability, *Archives of microbiology.* **188**, 411-9.

86. Mathews, M. M. & Krinsky, N. I. (1965) The relationship between carotenoid pigments and resistance to radiation in non-photosynthetic bacteria, *Photochemistry and photobiology.* **4**, 813-7.

87. Misra, H. S., Khairnar, N. P., Barik, A., Indira Priyadarsini, K., Mohan, H. & Apte, S. K. (2004) Pyrroloquinoline-quinone: a reactive oxygen species scavenger in bacteria, *FEBS Lett.* **578**, 26-30.

88. Khairnar, N. P., Misra, H. S. & Apte, S. K. (2003) Pyrroloquinoline-quinone synthesized in *Escherichia coli* by pyrroloquinoline-quinone synthase of Deinococcus radiodurans plays a role beyond mineral phosphate solubilization, *Biochemical and biophysical research communications.* **312**, 303-8.

89. Dose, K., Bieger-Dose, A., Labusch, M. & Gill, M. (1992) Survival in extreme dryness and DNA-single-strand breaks, *Advances in space research : the official journal of the Committee on Space Research.* **12**, 221-9.

90. Hansen, J. M., Go, Y. M. & Jones, D. P. (2006) Nuclear and mitochondrial compartmentation of oxidative stress and redox signaling, *Annual review of pharmacology and toxicology.* **46**, 215-34.

91. Mattimore, V. & Battista, J. R. (1996) Radioresistance of *Deinococcus radiodurans*: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation, *J Bacteriol.* **178**, 633-7.

92. de Groot, A., Dulermo, R., Ortet, P., Blanchard, L., Guerin, P., Fernandez, B., Vacherie, B., Dossat, C., Jolivet, E., Siguier, P., Chandler, M., Barakat, M., Dedieu, A., Barbe, V., Heulin, T., Sommer, S., Achouak, W. & Armengaud, J. (2009) Alliance of proteomics and genomics to unravel the specificities of Sahara bacterium *Deinococcus deserti*, *PLoS genetics.* **5**, e1000434.

93. Bauermeister, A., Moeller, R., Reitz, G., Sommer, S. & Rettberg, P. (2011) Effect of relative humidity on *Deinococcus radiodurans*' resistance to prolonged desiccation, heat, ionizing, germicidal, and environmentally relevant UV radiation, *Microb Ecol.* **61**, 715-22.

94. Moseley, B. E. & Evans, D. M. (1983) Isolation and properties of strains of *Micrococcus (Deinococcus) radiodurans* unable to excise ultraviolet light-induced pyrimidine dimers from DNA: evidence for two excision pathways, *Journal of general microbiology.* **129**, 2437-45.

95. Setlow, J. K. & Duggan, D. E. (1964) The Resistance of *Micrococcus Radiodurans* to Ultraviolet Radiation. I. Ultraviolet-Induced Lesions in the Cell's DNA, *Biochimica et biophysica acta.* **87**, 664-8.

96. Minton, K. W. (1994) DNA repair in the extremely radioresistant bacterium *Deinococcus radiodurans*, *Molecular microbiology.* **13**, 9-15.

97. Petit, C. & Sancar, A. (1999) Nucleotide excision repair: from *E. coli* to man, *Biochimie.* **81**, 15-25.

98. Alleva, J. L., Zuo, S., Hurwitz, J. & Doetsch, P. W. (2000) In vitro reconstitution of the *Schizosaccharomyces pombe* alternative excision repair pathway, *Biochemistry.* **39**, 2659-66.

99. Tanaka, M., Narumi, I., Funayama, T., Kikuchi, M., Watanabe, H., Matsunaga, T., Nikaido, O. & Yamamoto, K. (2005) Characterization of pathways dependent on the uvsE, uvrA1, or uvrA2 gene product for UV resistance in *Deinococcus radiodurans*, *J Bacteriol.* **187**, 3693-7.

100. Kuzminov, A. (1995) Collapse and repair of replication forks in *Escherichia coli*, *Molecular microbiology.* **16**, 373-84.

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

102. Tomasz, M., Lipman, R., Chowdary, D., Pawlak, J., Verdine, G. L. & Nakanishi, K. (1987) Isolation and structure of a covalent cross-link adduct between mitomycin C and DNA, *Science.* **235**, 1204-8.

103. Dronkert, M. L. & Kanaar, R. (2001) Repair of DNA interstrand cross-links, *Mutation research.* **486**, 217-47.

104. Koedrith, P. & Seo, Y. R. (2011) Enhancement of the efficacy of mitomycin C-mediated apoptosis in human colon cancer cells with RNAi-based thioredoxin reductase 1 deficiency, *Experimental and therapeutic medicine.* **2**, 873-878.

105. Gutman, P. D., Carroll, J. D., Masters, C. I. & Minton, K. W. (1994) Sequencing, targeted mutagenesis and expression of a recA gene required for the extreme radioresistance of *Deinococcus radiodurans*, *Gene.* **141**, 31-7.

106. Dillingham, M. S. & Kowalczykowski, S. C. (2008) RecBCD enzyme and the repair of double-stranded DNA breaks, *Microbiol Mol Biol Rev.* **72**, 642-71, Table of Contents.

107. Kowalczykowski, S. C., Dixon, D. A., Eggleston, A. K., Lauder, S. D. & Rehrauer, W. M. (1994) Biochemistry of homologous recombination in *Escherichia coli*, *Microbiol Rev.* **58**, 401-65.

108. Khairnar, N. P., Kamble, V. A. & Misra, H. S. (2008) RecBC enzyme overproduction affects UV and gamma radiation survival of *Deinococcus radiodurans*, *DNA repair.* **7**, 40-7.

109. Misra, H. S., Khairnar, N. P., Kota, S., Shrivastava, S., Joshi, V. P. & Apte, S. K. (2006) An exonuclease I-sensitive DNA repair pathway in *Deinococcus radiodurans*: a major determinant of radiation resistance, *Molecular microbiology.* **59**, 1308-16.

110. Bentchikou, E., Servant, P., Coste, G. & Sommer, S. (2010) A major role of the RecFOR pathway in DNA double-strand-break repair through ESDSA in *Deinococcus radiodurans*, *PLoS genetics.* **6**, e1000774.

111. Moseley, B. E., Mattingly, A. & Copland, H. J. (1972) Sensitization to radiation by loss of recombination ability in a temperature-sensitive DNA mutant of Micrococcus radiodurans held at its restrictive temperature, *Journal of general microbiology.* **72**, 329-38.

112. Bonacossa de Almeida, C., Coste, G., Sommer, S. & Bailone, A. (2002) Quantification of RecA protein in *Deinococcus radiodurans* reveals involvement of RecA, but not LexA, in its regulation, *Molecular genetics and genomics : MGG.* **268**, 28-41.

113. Narumi, I., Satoh, K., Kikuchi, M., Funayama, T., Yanagisawa, T., Kobayashi, Y., Watanabe, H. & Yamamoto, K. (2001) The LexA protein from *Deinococcus radiodurans* is not involved in RecA induction following gamma irradiation, *J Bacteriol.* **183**, 6951-6.

114. Kim, J. I. & Cox, M. M. (2002) The RecA proteins of *Deinococcus radiodurans* and *Escherichia coli* promote DNA strand exchange via inverse pathways, *Proc Natl Acad Sci U S A.* **99**, 7917-21.

115. Zou, Y., Liu, Y., Wu, X. & Shell, S. M. (2006) Functions of human replication protein A (RPA): from DNA replication to DNA damage and stress responses, *Journal of cellular physiology.* **208**, 267-73.

116. Sigal, N., Delius, H., Kornberg, T., Gefter, M. L. & Alberts, B. (1972) A DNA-unwinding protein isolated from *Escherichia coli*: its interaction with DNA and with DNA polymerases, *Proc Natl Acad Sci U S A.* **69**, 3537-41.

117. Weiner, J. H., Bertsch, L. L. & Kornberg, A. (1975) The deoxyribonucleic acid unwinding protein of *Escherichia coli*. Properties and functions in replication, *J Biol Chem.* **250**, 1972-80.

118. Murzin, A. G. (1993) OB(oligonucleotide/oligosaccharide binding)-fold: common structural and functional solution for non-homologous sequences, *The EMBO journal.* **12**, 861-7.

119. Shamoo, Y., Friedman, A. M., Parsons, M. R., Konigsberg, W. H. & Steitz, T. A. (1995) Crystal structure of a replication fork single-stranded DNA binding protein (T4 gp32) complexed to DNA, *Nature.* **376**, 362-6.

120. Lohman, T. M. & Ferrari, M. E. (1994) *Escherichia coli* single-stranded DNA-binding protein: multiple DNA-binding modes and cooperativities, *Annu Rev Biochem.* **63**, 527-70.

121. Lohman, T. M., Overman, L. B. & Datta, S. (1986) Salt-dependent changes in the DNA binding co-operativity of *Escherichia coli* single strand binding protein, *J Mol Biol.* **187**, 603-15.

122. Chrysogelos, S. & Griffith, J. (1982) *Escherichia coli* single-strand binding protein organizes single-stranded DNA in nucleosome-like units, *Proc Natl Acad Sci U S A.* **79**, 5803-7.

123. Bujalowski, W., Overman, L. B. & Lohman, T. M. (1988) Binding mode transitions of *Escherichia coli* single strand binding protein-single-stranded DNA complexes. Cation, anion, pH, and binding density effects, *J Biol Chem.* **263**, 4629-40.

124. Purnapatre, K. & Varshney, U. (1999) Cloning, over-expression and biochemical characterization of the single-stranded DNA binding protein from *Mycobacterium tuberculosis*, *European journal of biochemistry / FEBS.* **264**, 591-8.

125. Suck, D. (1997) Common fold, common function, common origin?, *Nat Struct Biol.* **4**, 161-5.

126. Lu, D. & Keck, J. L. (2008) Structural basis of *Escherichia coli* single-stranded DNA-binding protein stimulation of exonuclease I, *Proc Natl Acad Sci U S A.* **105**, 9169-74.

127. Hosoda, J. & Moise, H. (1978) Purification and physicochemical properties of limited proteolysis products of T4 helix destabilizing protein (gene 32 protein), *J Biol Chem.* **253**, 7547-58.

128. Savvides, S. N., Raghunathan, S., Futterer, K., Kozlov, A. G., Lohman, T. M. & Waksman, G. (2004) The C-terminal domain of full-length *E. coli* SSB is disordered even when bound to DNA, *Protein science : a publication of the Protein Society.* **13**, 1942-7.

129. Williams, K. R., Spicer, E. K., LoPresti, M. B., Guggenheimer, R. A. & Chase, J. W. (1983) Limited proteolysis studies on the *Escherichia coli* single-stranded DNA binding protein. Evidence for a functionally homologous domain in both the *Escherichia coli* and T4 DNA binding proteins, *J Biol Chem.* **258**, 3346-55.

130. Roy, R., Kozlov, A. G., Lohman, T. M. & Ha, T. (2007) Dynamic structural rearrangements between DNA binding modes of *E. coli* SSB protein, *J Mol Biol.* **369**, 1244-57.

131. Chase, J. W., L'Italien, J. J., Murphy, J. B., Spicer, E. K. & Williams, K. R. (1984) Characterization of the *Escherichia coli* SSB-113 mutant single-stranded DNA-binding protein. Cloning of the gene, DNA and protein sequence analysis, high pressure liquid chromatography peptide mapping, and DNA-binding studies, *J Biol Chem.* **259**, 805-14.

132. Genschel, J., Curth, U. & Urbanke, C. (2000) Interaction of *E. coli* single-stranded DNA binding protein (SSB) with exonuclease I. The carboxy-terminus of SSB is the recognition site for the nuclease, *Biological chemistry.* **381**, 183-92.

133. Greenberg, J., Berends, L. J., Donch, J. & Green, M. H. (1974) exrB: a malB-linked gene in *Escherichia coli* B involved in sensitivity to radiation and filament formation, *Genetical research.* **23**, 175-84.

134. Greenberg, J. & Donch, J. (1974) Sensitivity to elevated temperatures in exrB strains of *Escherichia coli*, *Mutation research.* **25**, 403-5.

135. Curth, U., Genschel, J., Urbanke, C. & Greipel, J. (1996) In vitro and in vivo function of the C-terminus of *Escherichia coli* single-stranded DNA binding protein, *Nucleic Acids Res.* **24**, 2706-11.

136. Mijakovic, I., Petranovic, D., Macek, B., Cepo, T., Mann, M., Davies, J., Jensen, P. R. & Vujaklija, D. (2006) Bacterial single-stranded DNA-binding proteins are phosphorylated on tyrosine, *Nucleic Acids Res.* **34**, 1588-96.

137. Yuzhakov, A., Kelman, Z. & O'Donnell, M. (1999) Trading places on DNA--a three-point switch underlies primer handoff from primase to the replicative DNA polymerase, *Cell.* **96**, 153-63.

138. Rowen, L. & Kornberg, A. (1978) Primase, the dnaG protein of *Escherichia coli*. An enzyme which starts DNA chains, *J Biol Chem.* **253**, 758-64.

139. Kaguni, J. M. & Kornberg, A. (1984) Replication initiated at the origin (oriC) of the *E. coli* chromosome reconstituted with purified enzymes, *Cell.* **38**, 183-90.

140. Shereda, R. D., Bernstein, D. A. & Keck, J. L. (2007) A central role for SSB in *Escherichia coli* RecQ DNA helicase function, *J Biol Chem.* **282**, 19247-58.

141. Lovett, S. T. & Clark, A. J. (1984) Genetic analysis of the recJ gene of *Escherichia coli* K-12, *J Bacteriol.* **157**, 190-6.

142. Lovett, S. T. & Kolodner, R. D. (1989) Identification and purification of a single-stranded-DNA-specific exonuclease encoded by the recJ gene of *Escherichia coli*, *Proc Natl Acad Sci U S A.* **86**, 2627-31.

143. Han, E. S., Cooper, D. L., Persky, N. S., Sutera, V. A., Jr., Whitaker, R. D., Montello, M. L. & Lovett, S. T. (2006) RecJ exonuclease: substrates, products and interaction with SSB, *Nucleic Acids Res.* **34**, 1084-91.

144. Meddows, T. R., Savory, A. P. & Lloyd, R. G. (2004) RecG helicase promotes DNA double-strand break repair, *Molecular microbiology.* **52**, 119-32.

145. Ishioka, K., Iwasaki, H. & Shinagawa, H. (1997) Roles of the recG gene product of *Escherichia coli* in recombination repair: effects of the delta recG mutation on cell division and chromosome partition, *Genes & genetic systems.* **72**, 91-9.

146. Tanaka, T. & Masai, H. (2006) Stabilization of a stalled replication fork by concerted actions of two helicases, *J Biol Chem.* **281**, 3484-93.

147. Slocum, S. L., Buss, J. A., Kimura, Y. & Bianco, P. R. (2007) Characterization of the ATPase activity of the *Escherichia coli* RecG protein reveals that the preferred cofactor is negatively supercoiled DNA, *J Mol Biol.* **367**, 647-64.

148. Lecointe, F., Serena, C., Velten, M., Costes, A., McGovern, S., Meile, J. C., Errington, J., Ehrlich, S. D., Noirot, P. & Polard, P. (2007) Anticipating chromosomal replication fork arrest: SSB targets repair DNA helicases to active forks, *The EMBO journal.* **26**, 4239-51.

149. Kantake, N., Madiraju, M. V., Sugiyama, T. & Kowalczykowski, S. C. (2002) *Escherichia coli* RecO protein anneals ssDNA complexed with its cognate ssDNA-binding protein: A common step in genetic recombination, *Proc Natl Acad Sci U S A.* **99**, 15327-32.

150. Hobbs, M. D., Sakai, A. & Cox, M. M. (2007) SSB protein limits RecOR binding onto single-stranded DNA, *J Biol Chem.* **282**, 11058-67.

151. Register, J. C., 3rd & Griffith, J. (1985) The direction of RecA protein assembly onto single strand DNA is the same as the direction of strand assimilation during strand exchange, *J Biol Chem.* **260**, 12308-12.

152. Flory, S. S., Tsang, J., Muniyappa, K., Bianchi, M., Gonda, D., Kahn, R., Azhderian, E., Egner, C., Shaner, S. & Radding, C. M. (1984) Intermediates in homologous pairing promoted by RecA protein and correlations of recombination in vitro and in vivo, *Cold Spring Harbor symposia on quantitative biology.* **49**, 513-23.

153. Lavery, P. E. & Kowalczykowski, S. C. (1992) A postsynaptic role for single-stranded DNA-binding protein in recA protein-promoted DNA strand exchange, *J Biol Chem.* **267**, 9315-20.

154. Bhaduri, T., Bagui, T. K., Sikder, D. & Nagaraja, V. (1998) DNA topoisomerase I from *Mycobacterium smegmatis*. An enzyme with distinct features, *J Biol Chem.* **273**, 13925-32.

155. Bhaduri, T., Sikder, D. & Nagaraja, V. (1998) Sequence specific interaction of *Mycobacterium smegmatis* topoisomerase I with duplex DNA, *Nucleic Acids Res.* **26**, 1668-74.

156. de la Tour, C. B., Passot, F. M., Toueille, M., Mirabella, B., Guerin, P., Blanchard, L., Servant, P., de Groot, A., Sommer, S. & Armengaud, J. (2013) Comparative proteomics reveals key proteins recruited at the nucleoid of *Deinococcus* after irradiation-induced DNA damage, *Proteomics.* **13**, 3457-69.

157. Norais, C. A., Chitteni-Pattu, S., Wood, E. A., Inman, R. B. & Cox, M. M. (2009) DdrB protein, an alternative *Deinococcus radiodurans* SSB induced by ionizing radiation, *J Biol Chem.* **284**, 21402-11.

158. Xu, G., Lu, H., Wang, L., Chen, H., Xu, Z., Hu, Y., Tian, B. & Hua, Y. (2010) DdrB stimulates single-stranded DNA annealing and facilitates RecA-independent DNA repair in *Deinococcus radiodurans*, *DNA repair.* **9**, 805-12.

159. Lindner, C., Nijland, R., van Hartskamp, M., Bron, S., Hamoen, L. W. & Kuipers, O. P. (2004) Differential expression of two paralogous genes of *Bacillus subtilis* encoding single-stranded DNA binding protein, *J Bacteriol.* **186**, 1097-105.

160. Paradzik, T., Ivic, N., Filic, Z., Manjasetty, B. A., Herron, P., Luic, M. & Vujaklija, D. (2013) Structure-function relationships of two paralogous single-stranded DNA-binding proteins from *Streptomyces coelicolor*: implication of SsbB in chromosome segregation during sporulation, *Nucleic Acids Res.* **41**, 3659-72.

161. Witte, G., Urbanke, C. & Curth, U. (2005) Single-stranded DNA-binding protein of *Deinococcus radiodurans*: a biophysical characterization, *Nucleic Acids Res.* **33**, 1662-70.

162. Tanaka, M., Earl, A. M., Howell, H. A., Park, M. J., Eisen, J. A., Peterson, S. N. & Battista, J. R. (2004) Analysis of *Deinococcus radiodurans*'s transcriptional response to ionizing radiation and desiccation reveals novel proteins that contribute to extreme radioresistance, *Genetics.* **168**, 21-33.

163. Basu, B. & Apte, S. K. (2012) Gamma radiation-induced proteome of *Deinococcus radiodurans* primarily targets DNA repair and oxidative stress alleviation, *Mol Cell Proteomics.* **11**, M111 011734.

164. Ujaoney, A. K., Potnis, A. A., Kane, P., Mukhopadhyaya, R. & Apte, S. K. (2010) Radiation desiccation response motif-like sequences are involved in transcriptional activation of the Deinococcal ssb gene by ionizing radiation but not by desiccation, *J Bacteriol.* **192**, 5637-44.

165. Lu, H., Gao, G., Xu, G., Fan, L., Yin, L., Shen, B. & Hua, Y. (2009) *Deinococcus radiodurans* PprI switches on DNA damage response and cellular survival networks after radiation damage, *Mol Cell Proteomics.* **8**, 481-94.

166. Yin, L., Wang, L., Lu, H., Xu, G., Chen, H., Zhan, H., Tian, B. & Hua, Y. (2010) DRA0336, another OxyR homolog, involved in the antioxidation mechanisms in *Deinococcus radiodurans*, *Journal of microbiology.* **48**, 473-9.

167. Mattimore, V., Udupa, K. S., Berne, G. A. & Battista, J. R. (1995) Genetic characterization of forty ionizing radiation-sensitive strains of *Deinococcus radiodurans*: linkage information from transformation, *J Bacteriol.* **177**, 5232-7.

168. Birnboim, H. C. & Doly, J. (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA, *Nucleic Acids Res.* **7**, 1513-23.

169. Kim, J. I., Sharma, A. K., Abbott, S. N., Wood, E. A., Dwyer, D. W., Jambura, A., Minton, K. W., Inman, R. B., Daly, M. J. & Cox, M. M. (2002) RecA Protein from the extremely radioresistant bacterium *Deinococcus radiodurans*: expression, purification, and characterization, *J Bacteriol.* **184**, 1649-60.

170. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) Protein measurement with the Folin phenol reagent, *J Biol Chem.* **193**, 265-75.

171. Yang, S. H., Zhou, R., Campbell, J., Chen, J., Ha, T. & Paull, T. T. (2013) The SOSS1 single-stranded DNA binding complex promotes DNA end resection in concert with Exo1, *The EMBO journal.* **32**, 126-39.

172. Esser, D., Rudolph, R., Jaenicke, R. & Bohm, G. (1999) The HU protein from *Thermotoga maritima*: recombinant expression, purification and physicochemical characterization of an extremely hyperthermophilic DNA-binding protein, *J Mol Biol.* **291**, 1135-46.

173. Iyer, V. N. & Szybalski, W. (1963) A Molecular Mechanism of Mitomycin Action: Linking of Complementary DNA Strands, *Proc Natl Acad Sci U S A.* **50**, 355-62.

174. Cabiscol, E., Tamarit, J. & Ros, J. (2000) Oxidative stress in bacteria and protein damage by reactive oxygen species, *International microbiology : the official journal of the Spanish Society for Microbiology.* **3**, 3-8.

175. Carroll, J. D., Daly, M. J. & Minton, K. W. (1996) Expression of recA in *Deinococcus radiodurans*, *J Bacteriol.* **178**, 130-5.

176. Lipton, M. S., Pasa-Tolic, L., Anderson, G. A., Anderson, D. J., Auberry, D. L., Battista, J. R., Daly, M. J., Fredrickson, J., Hixson, K. K., Kostandarithes, H., Masselon, C., Markillie, L. M., Moore, R. J., Romine, M. F., Shen, Y., Stritmatter, E., Tolic, N., Udseth,

H. R., Venkateswaran, A., Wong, K. K., Zhao, R. & Smith, R. D. (2002) Global analysis of the *Deinococcus radiodurans* proteome by using accurate mass tags, *Proc Natl Acad Sci U S A.* **99**, 11049-54.

177. Ali, S. S., Beckett, E., Bae, S. J. & Navarre, W. W. (2011) The 5.5 protein of phage T7 inhibits H-NS through interactions with the central oligomerization domain, *J Bacteriol.* **193**, 4881-92.

178. Bhowmick, T., Ghosh, S., Dixit, K., Ganesan, V., Ramagopal, U. A., Dey, D., Sarma, S. P., Ramakumar, S. & Nagaraja, V. (2014) Targeting *Mycobacterium tuberculosis* nucleoid-associated protein HU with structure-based inhibitors, *Nature communications.* **5**, 4124.

179. Basu, B. (2010) Radiation resistance of *Deinococcus radiodurans*: Role of chaperones and protease in surviving radiation stress. in *Molecuar Biology* pp. 1-276, Mumbai University, Mumbai.

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Radiation Desiccation Response Motif-Like Sequences Are Involved in Transcriptional Activation of the Deinococcal *ssb* Gene by Ionizing Radiation but Not by Desiccation▽

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Single-stranded-DNA binding protein (SSB) levels during poststress recovery of *Deinococcus radiodurans* were significantly enhanced by ^{60}Co gamma rays or mitomycin C treatment but not by exposure to UV rays, hydrogen peroxide (H_2O_2), or desiccation. Addition of rifampin prior to postirradiation recovery blocked such induction. *In silico* analysis of the *ssb* promoter region revealed a 17-bp palindromic radiation/desiccation response motif (RDRM1) at bp −114 to −98 and a somewhat similar sequence (RDRM2) at bp −213 to −197, upstream of the *ssb* open reading frame. Involvement of these *cis* elements in radiation-responsive *ssb* gene expression was assessed by constructing transcriptional fusions of edited versions of the *ssb* promoter region with a nonspecific acid phosphatase encoding reporter gene, *phoN*. Recombinant *D. radiodurans* strains carrying such constructs clearly revealed (i) transcriptional induction of the *ssb* promoter upon irradiation and mitomycin C treatment but not upon UV or H_2O_2 treatment and (ii) involvement of both RDRM-like sequences in such activation of SSB expression, in an additive manner.

Tolerance to high doses of ionizing radiation (X rays and gamma rays), which cause extensive and lethal DNA damage, is rare among life forms. However, species of the Gram-positive, pink-orange aerobic bacterium *Deinococcus* have been known to survive exposure to extremely high doses of ionizing radiation, UV rays, mitomycin C, desiccation, and other DNA-damaging agents (4, 30). Members of the family *Deinococcaceae*, comprising over 30 species, inhabit diverse environments and survive 5 kGy of ^{60}Co gamma rays without any loss of viability (31). Some of the strains are known to survive doses as high as even 15 kGy or more (13). The phenomena underlying such extremophilic behavior of deinococci, though not entirely clear, fall into three major categories. These are (i) a unique condensed organization of the genome, which minimizes DNA damage and facilitates rapid postirradiation repair (9, 11, 23, 43); (ii) highly proficient, regular, and novel DNA repair mechanisms (6, 8) aided by proteins unique to deinococci (6, 38); and (iii) very capable enzymatic/nonenzymatic cleaning systems to scavenge reactive oxygen species (ROS) and protect proteins from oxidative damage (9, 10) or to degrade and resynthesize damaged proteins (22) in order to quickly alleviate the radiation toxicity and restore cellular homeostasis. Consequently, the conventional enzymes responsible for postirradiation recovery (PIR) survive and function with far better efficiency in deinococci (26). New mechanisms continue to be elaborated (20, 22, 42).

Genomes of at least three highly radioresistant *Deinococcus* spp. have already been completely sequenced (14, 26, 39). These species are (i) *D. radiodurans*, the species of the first prototype strain isolated from irradiated meat cans 50 years ago and the most studied so far (1); (ii) the moderately thermophilic *D. geothermalis*, isolated from a hot spring in Italy (17); and (iii) the most recent, *D. deserti*, obtained from surface sands of the Sahara desert (13). *In silico* analyses of the genomes of these species have defined a minimal set of genes required for extreme radiation/desiccation resistance (RDR) in the genus *Deinococcus* (14, 26). Comparative genomics supplemented by microarray and proteomic analyses have revealed enhanced expression of several genes in *Deinococcus* spp. immediately following irradiation (24, 25, 38). As expected, this set is dominated by genes related to DNA end protection, replication, recombination, and repair, but it also includes genes encoding chaperones, proteases, RNA binding proteins, RNA ligases, transcription factors, membrane transporters, Krebs cycle enzymes, transposases, superoxide dismutases/thioredoxin/peroxidases, and even tellurium resistance and plant LEA-like proteins (5, 10, 14, 19, 20, 25, 26, 29, 32, 38).

Mechanisms which facilitate radiation-induced gene expression have been investigated for a very few selected genes (such as *recA*) in deinococci and remain poorly understood. *D. radiodurans recA* expression is induced by the PprI protein, which, however, does not bind the *recA* promoter (15, 18, 21). LexA, the well-known repressor of *recA* expression in most bacteria, is not induced upon irradiation in *D. radiodurans* and does not regulate postirradiation *recA* induction (33). In most cases, the radiation-enhanced gene expression appears to be due to transcriptional activation (24, 25), but the corresponding regulatory *cis* elements and *trans*-acting proteins have remained largely unexplored so far. Critical examination of deinococcal genomes recently identified a 17-bp palindromic sequence (**T**, **T**, A/C, T/C, **G**, T/C, N, N, **T**/**A**, N, A, A/G, **C**,

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TABLE 1. Plasmids and primers used in the study

Plasmid or primer	Description[a]	Reference, source, or comment
Plasmids		
pRAD1	*E. coli-D. radiodurans* shuttle vector (copy no., 7–10 per cell in *D. radiodurans*)	28
pRN1	pRAD1 with *phoN* cloned at NdeI and BamHI sites; Cb^r Cm^r	Lab collection
pTZ57R/T	PCR product cloning vector (2,886 bp, Ap^r) for blue-white selection of transformants	MBI Fermentas
pTAPssb102	102-bp sequence upstream of *D. radiodurans ssb* cloned in pTZ57R/T	This study
pSN2	pRN1 with 112-bp fragment from pTAPssb102 subcloned at NruI and NdeI sites	This study
pSN3	pRN1 with 132-bp sequence upstream of *D. radiodurans ssb* ORF cloned at XbaI and NdeI sites	This study
pSN4	pRN1 with 351-bp sequence upstream of *D. radiodurans ssb* ORF cloned at XbaI and NdeI sites	This study
Primers		
P1	5′-CCGAGAAGGATTACAATCTAGAACG-3′	XbaI
P2	5′-CGGAGTGGAAGATCTAGAAGGCCTG-3′	XbaI
P3	5′-GCGAGCTCATAATTGACTCTGCTTGTTACTATCTAGTG-3′	SacI
P4	5′-CATGCCTCGGGCCATATGAAATTCT-3′	NdeI
P5	5′-GGAGCGGATAACAATTTCACACA-3′	P5 and P6 flank multiple-cloning site, 58 bp on either side in pRad1
P6	5′-AACGCGGCTGCAAGAATGGTA-3′	

[a] The underlined sequence corresponds to the restriction site incorporated in the primer.

G/A, T/G, **A**, **A**) upstream of several radiation-induced genes in all three *Deinococcus* spp. (14, 26). The most conserved nucleotides are shown in bold, while the center of dyad symmetry lies around the ninth nucleotide, which is T/A. The sequence, first described by Makarova et al. as a radiation/desiccation response motif (RDRM), was found upstream of 29 genes in *D. radiodurans* and 25 genes in *D. geothermalis* (26). Subsequently, the motif has also been reported upstream of 25 genes in *D. deserti* by de Groot et al., (14), who have preferred to describe it as radiation response motif (RRM). The radiation and desiccation resistance (RDR) regulon common to all three *Deinococcus* spp. thus includes about 25 genes, all of which possess RDRM sequences upstream of their open reading frames (ORFs). Prominent genes/operons in this set include several DNA replication/recombination/repair-related genes, such as *ssb*, *gyrA* and *gyrB*, *cinA*/*ligT*/*recA*, *uvrA*, *uvrB* and *uvrD*, *mutS*, *recQ*, *ruvB*, *pprA*, *ddrA*, *ddrB*, *ddrD*, and others (6, 14, 26). In *D. radiodurans* and in *D. deserti*, for which transcriptome data are available, all the genes of the RDR are also upregulated during postirradiation recovery (14). Strangely, however, the involvement of RDRM sequences *per se* in desiccation/radiation induction of deinococcal genes has never been verified experimentally.

The present study assessed the role of RDRM-like sequences in radiation-induced gene expression, taking the single-stranded-DNA binding protein (SSB)-encoding gene, *ssb*, as a test case. The *ssb* gene in *D. radiodurans* harbors two RDRM-like sequences, RDRM1 and RDRM2, located between bp −114 and −98 and bp −213 and −197, respectively (16, 39; http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=gdr). Using truncated versions of the *ssb* promoter carrying none, one, or both of the RDRM-like sequences fused to the reporter gene *phoN*, we have demonstrated that both RDRM sequences are involved in radiation-induced *ssb* expression, in an additive way. Our data also show that desiccation or exposure to UV or hydrogen peroxide treatment does not influence *ssb* expression in *D. radiodurans*.

MATERIALS AND METHODS

Bacterial strains and growth condition. *Deinococcus radiodurans* strain R1 was maintained in TGY (1% tryptone, 0.1% glucose, 0.5% yeast extract) medium at 32°C under agitation at 150 rpm. *Escherichia coli* strain DH5α [F^- *recA41 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44 relA lacU169*] was maintained in Luria-Bertani (LB) medium at 37°C under agitation at 150 rpm. Bacterial growth was measured spectrophotometrically as turbidity (optical density at 600 nm [OD_{600}]). The antibiotics used for selection of recombinants were chloramphenicol (3 μg ml^{-1}) for *D. radiodurans* and carbenicillin (100 μg ml^{-1}) for *E. coli* DH5α.

PCR amplification, cloning, and transformation. The pRN1 vector, generated previously in our laboratory, contains a promoterless *phoN* gene (Table 1) and was used for cloning of all the putative promoter fragments. Genomic DNA isolation from *D. radiodurans* and plasmid isolation from *E. coli* were carried out as described previously (28, 37). The primers used for PCR were designed based on the corrected published sequences of the *ssb* gene (16) and the *Deinococcus* genome (39; http://cmr.jcvi.org/cgi-bin/CMR/GenomePage.cgi?org=gdr). All PCR products were purified using a QiaexII gel extraction kit (Qiagen, Hilden, Germany). For construction of plasmids pSN3 and pSN4, the 132-bp and 351-bp DNA sequences upstream of the *ssb* open reading frame (ORF) (see Fig. 3b and c) were PCR amplified using primers shown in Table 1. The PCR products were gel purified, restriction digested with XbaI-NdeI, and ligated at same sites in pRN1. Plasmid pSN2 was generated in two steps. First, 102 bp of DNA upstream of the *ssb* ORF was PCR amplified using primers P3 and P4 and ligated in the pTZ57R/T PCR product cloning vector (Table 1), yielding pTAPssb102. Next, plasmid pTAPssb102 was restriction digested with SmaI-NdeI to release the 112-bp insert, which was gel purified and ligated to pRN1 at NruI-NdeI sites to obtain pSN2. All the plasmid constructs were transformed in *E. coli* DH5α cells and screened on plates containing carbenicillin (100 μg ml^{-1}). Plasmids isolated from *E. coli* DH5α were used to transform *D. radiodurans* cells as described earlier (28). All recombinant clones were also screened by colony PCR and confirmed by sequencing using primers P5 and P6 (Table 1). Deinococcal transformants expressing the *phoN* reporter gene were screened on histochemical plates (2, 36) containing phenolphthalein diphosphate (PDP) (1 mg ml^{-1}), methyl green (MG) (10 μg ml^{-1}), and chloramphenicol (Cm) (3 μg ml^{-1}). The PhoN-positive colonies exhibited a green color.

Treatment with various stresses. Wild-type and recombinant *D. radiodurans* cells (carrying plasmid pSN2, pSN3, pSN4, or pRN1) were grown overnight at 32°C to an OD_{600} of 4 to 5/ml and subjected to one of the following stress treatments: (i) 2 to 8 kGy of gamma radiation from a ^{60}Co source (GC220; Atomic Energy of Canada Limited, Canada) (0.36 kGy h^{-1}), (ii) 20 μg ml^{-1} of mitomycin C (Sigma, St. Louis, MO) for 15 min at 32°C, (iii) 50 or 100 mM hydrogen peroxide (Ranchem, New Delhi, India) for 1 h at 32°C, or (iv) 2.5 or 5 kJ m^{-2} of UV (Philips) (5 J m^{-2} s^{-1}). UV stress was applied to 4 ml of culture

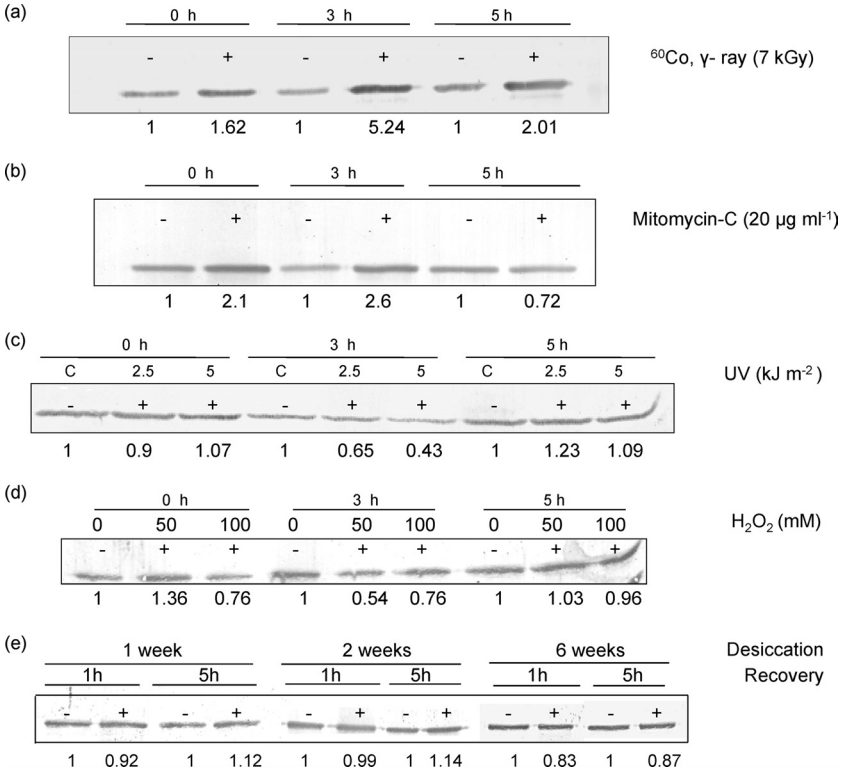

FIG. 1. Effect of DNA-damaging agents on deinococcal SSB levels. Stationary-phase deinococcal cells were exposed to the specified dose or concentration of either gamma rays (a), mitomycin C for 15 min (b), UV rays (c), or H_2O_2 for 1 h (d) or were dehydrated in a desiccator using fused calcium chloride as desiccant for the specified duration (e). After the stress, cells were allowed to recover in TGY medium, and proteins were extracted at the specified time points during poststress recovery. Equal amounts of proteins (30 μg) were electrophoretically resolved by 12% SDS-PAGE and electroblotted on nitrocellulose membrane. SSB levels in stressed (+) and unstressed (−) samples were immunodetected using anti-*D. radiodurans* SSB antibody and quantitated. The numbers below the lanes depict quantitation of SSB levels by densitometry.

spread in a 9-cm sterile petri plate to form a <1-mm layer and to minimize absorption by the TGY medium. For desiccation stress, *D. radiodurans* cells grown overnight were filtered and subjected to 1, 2, or 6 weeks of desiccation using fused calcium chloride in desiccators stored at 23°C. Following stress treatments, cells were washed three times with fresh TGY medium, inoculated in TGY at a final OD_{600} of 0.5, and allowed to recover. Periodically, samples were removed for various analyses and either used directly for biochemical assays or snap-frozen in liquid nitrogen and stored at −70°C for Western blots or zymograms.

Western blotting and immunodetection of SSB. For assessment of transcriptional activation of *ssb* expression, wild-type *D. radiodurans* cells grown overnight (OD_{600} = 5 ml^{-1}) were divided into two sets. To one set, rifampin (50 μg ml^{-1}) was added prior to gamma radiation of 7 kGy and washed off before PIR; to the second set, rifampin was added after irradiation but before PIR of cells. An additional treatment had TGY medium with rifampin (50 μg ml^{-1}) which was irradiated separately and added during PIR of irradiated cells. All treatments had their corresponding unirradiated controls.

For quantitation of SSB levels, proteins were extracted from wild-type *D. radiodurans* after 3 h of poststress recovery. Protein extracts (30 to 100 μg) from control or stressed samples were resolved by 12% SDS-PAGE and electroblotted on a nitrocellulose membrane. The primary antibody used was anti-*D. radiodurans* SSB antibody at a 1:200 dilution and incubated overnight, while the secondary antibody used was anti-rabbit IgG (Sigma, St. Louis, MO) coupled to alkaline phosphatase at a 1:10,000 dilution and incubated for 1.5 to 2 h. The blot was developed using the substrate nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Roche Biochemicals, Germany).

Assays for reporter gene activity. *phoN* was used as a reporter gene for the first time in this study. PhoN is a periplasmic nonspecific acid phosphatase that cleaves organic phosphate to release phosphoric acid, optimally at pH 5 to 7 (7). PhoN activity expressed from the P*ssb* promoter was monitored in the following two ways: (i) by zymogram analyses of stressed cells after 3 h of PIR, as described earlier (2, 36), with activity staining of PhoN protein on zymograms carried out using NBT-BCIP and (ii) by spectrophotometric analysis of cell-bound PhoN activity as *p*-nitrophenol (pNP) released from *p*-nitrophenylphosphate (pNPP) (Sisco Research Laboratories, Mumbai, India) at 405 nm. Acid phosphatase enzyme activity of cells is reported as nmol of pNP liberated min^{-1} OD_{600} unit^{-1} (7).

RESULTS

Effects of DNA-damaging agents on deinococcal *ssb* expression. *D. radiodurans* cells exposed to gamma rays (7 kGy) or mitomycin C (20 μg ml^{-1} for 15 min) showed significant induction of immunodetectable SSB levels on Western blots during poststress recovery (Fig. 1a and 1b). The increase in SSB levels displayed a time dependence, with maximum enhancement observed at 3 h of poststress recovery following exposure to both stressors (Fig. 1a and b). Exposure to high doses of UV rays (2.5 to 5 kJ m^{-2}) or hydrogen peroxide (50 to 100 mM) did not influence cellular SSB levels in *D. radiodurans* (Fig. 1c and d). Prolonged desiccation of 1 to 6 weeks also did not affect SSB levels (Fig. 1e).

Effect of gamma irradiation on *ssb* expression in *D. radiodurans*. A distinct radiation dose-dependent increase in SSB levels, compared to those in the unirradiated controls, was observed during postirradiation recovery in *D. radiodurans*, resulting in 6.01-fold-higher SSB levels at a 7-kGy dose after 3 h of postirradiation recovery (PIR) (Fig. 2a). The observed radiation-induced SSB enhancement at 7 kGy was abolished if rifampin was added during PIR (Fig. 2b, lanes 4 and 5) but was

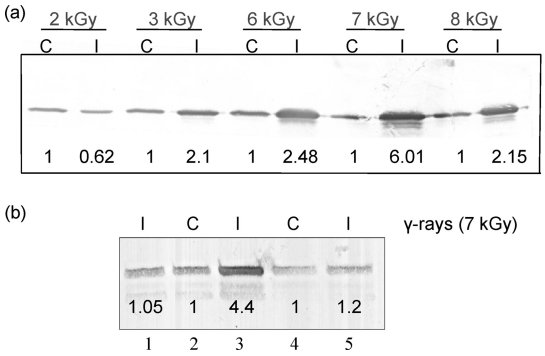

FIG. 2. Effect of ionizing radiation on deinococcal SSB levels. (a) Stationary-phase *D. radiodurans* cells were exposed to specified dose of ^{60}Co gamma rays. (b) Cells were irradiated (7 kGy) in the presence of 50 μg ml^{-1} of rifampin, which was either added during irradiation and washed off before recovery (lanes 2 and 3), added during PIR (lanes 4 and 5), or irradiated (7 kGy) (I) and added during PIR (lane 1), and compared with unirradiated control (C) cells. Posttreatment cells were allowed to recover in TGY medium for 3 h. Protein extracts (100-μg blot [a] or 30-μg blot) were electrophoretically resolved. Other details were as described for Fig. 1.

seen in cells irradiated in the presence of rifampin but recovered in the absence of transcriptional inhibitor (Fig. 2b, lane 3). Addition of irradiated rifampin during PIR also inhibited the radiation-induced SSB enhancement (Fig. 2b, lane 1), indicating that irradiation did not inactivate rifampin.

Bioinformatic analysis of DNA sequence upstream of the deinococcal *ssb* gene. The *ssb* gene in *D. radiodurans* is located 119 bp downstream of the annotated *rpsF* gene and is followed by the *rpsR* gene, located 56 bp downstream of *ssb* (Fig. 3a). *In silico* analysis of the 351-bp region upstream of the *ssb* ORF using BPROM software (Softberry) predicted the putative −10 and −35 promoter-like sequences, while a manual sequence search revealed the Shine Dalgarno (SD) sequence upstream of the *ssb* ORF (Fig. 3b). A 17-bp predicted conserved radiation/desiccation response motif (5′-TTATGTCAT TGACATAA-3′) was observed between bp −114 and −98 (RDRM1), and a not-as-conserved but similar motif (5′-AAC CGCCATCGCCAGCA-3′) was observed between bp −213 and −197 (RDRM2) upstream of the start of the *ssb* ORF (Fig. 3b). This places RDRM1 in the intergenic region between the *rpsF* and *ssb* genes and RDRM2 within the *rpsF* gene, 74 bp upstream of the stop codon (Fig. 3b and c).

Construction of P*ssb-phoN* transcriptional fusions. In order to assess the possible role played by the two RDRM-like sequences in regulation of *ssb* expression, transcriptional fusions of RDRM carrying DNA sequences were constructed with the *phoN* gene (Fig. 3c), which encodes a nonspecific acid phosphatase. For this, we used an existing plasmid, pRN1 (Table 1). Specified lengths of *ssb* promoter regions were cloned in pRN1 (as described in Materials and Methods) to generate recombinant plasmids pSN2, pSN3, and pSN4 (Fig. 3c; Table 1). The plasmid pSN4 carried both RDRM1 and RDRM2, pSN3 carried only RDRM1, and pSN2 carried only the last 5 bases of the RDRM1 sequence (Fig. 3b and c).

Assessment of reporter (PhoN) activity expressed from the deinococcal *ssb* promoter. Differences among various clones were visualized using activity staining on zymograms. In-gel enzyme activity of PhoN in clone pSN4 showed strong induc-

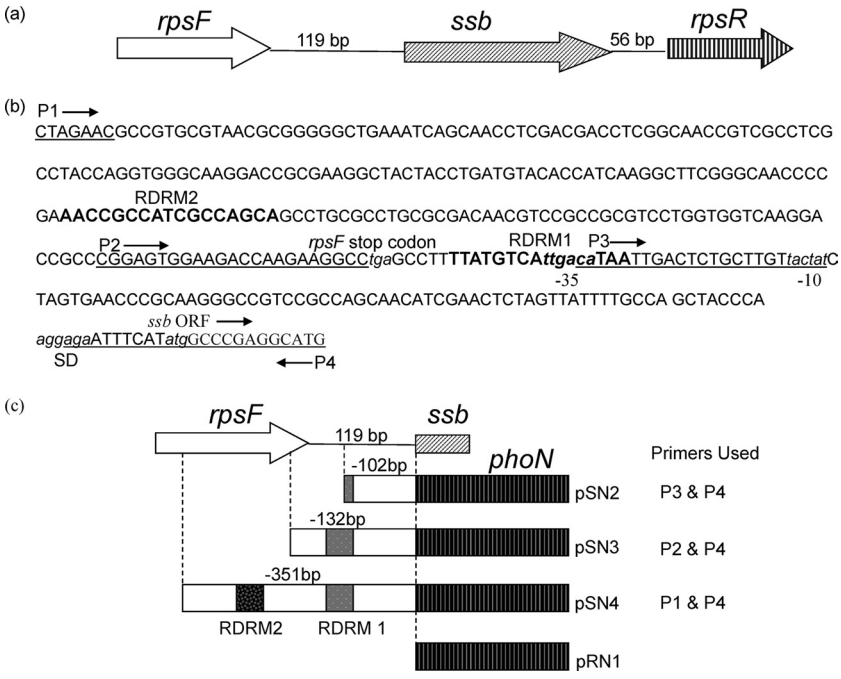

FIG. 3. Organization of the *ssb* gene in *D. radiodurans* and various constructs. (a) Genomic location of the *ssb* gene in *D. radiodurans.* (b) Nucleotide sequence of the putative promoter region of *ssb* from the *D. radiodurans* genome, showing various *cis* elements. RDRM1 and RDRM2 sequences are shown in bold. A putative SD sequence, −10- and −35-like sequences, the *rpsF* stop codon, and the *ssb* start codon are italicized and labeled. Primer sequences are underlined. The first codon of the *D. radiodurans ssb* ORF is shown. (c) Schematic representation of various P*ssb-phoN* transcriptional fusions and the primers used.

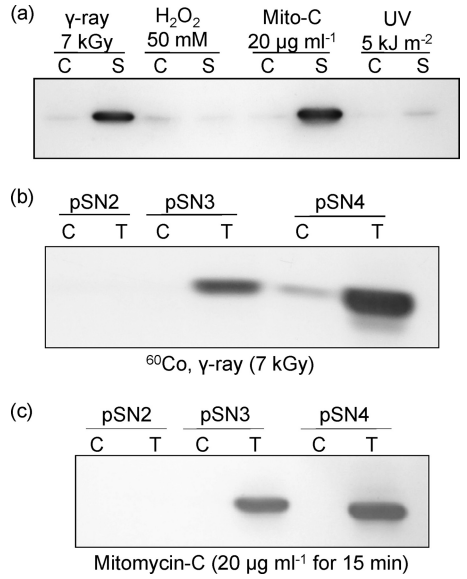

FIG. 4. Zymogram analysis of PhoN reporter activity expressed from the deinococcal *ssb* promoter during poststress recovery. (a) The recombinant *D. radiodurans* strain carrying the construct pSN4 was exposed to different stresses and allowed to recover in fresh TGY medium for 3 h under the usual growth conditions. Proteins were extracted in nonreducing buffer and electrophoretically resolved by 12% SDS-PAGE. Activity bands were developed by incubating the gel with NBT-BCIP in acetate buffer. (b and c) Recombinant *D. radiodurans* strains carrying either pSN2, pSN3, or pSN4 were exposed to 7 kGy of ^{60}Co gamma rays or 20 μg ml^{-1} of mitomycin C for 15 min. Other details were as described for panel a.

tion of the reporter gene after exposure of cells to gamma rays or mitomycin C but not upon exposure to UV or H_2O_2 stress (Fig. 4a). The gamma ray/mitomycin C induction of various P*ssb-phoN* constructs was rigorously assessed further (Fig. 4b and c). For both the stresses, the highest in-gel enzyme activity was observed in clone pSN4, followed by clone pSN3, while only basal-level activity was seen in clone pSN2 (Fig. 4b and c). The relative induction of P*ssb*-driven PhoN expression in various clones was further confirmed and quantitated by spectrophotometric analysis of PhoN enzyme activity, using pNPP as the substrate (Fig. 5). The reporter gene activity (PhoN) in both clones pSN3 and pSN4 increased steadily with time. For both gamma rays and mitomycin C, clone pSN4 cells showed much higher activity (~6- to 8-fold after 5 h) than pSN3 (~4- to 5-fold after 5 h) when equivalent cells were tested (Fig. 5). Clone pSN2 showed only basal PhoN activity and did not respond to irradiation or mitomycin C treatment.

DISCUSSION

The single-stranded-DNA binding protein (SSB), in conjunction with the RecA protein, is involved in all important DNA-related cellular activities, such as replication, transcription, recombination, and repair, in bacteria. Several earlier studies have shown that RecA is normally expressed at a low basal level in *D. radiodurans* but that its levels significantly increase during recovery from radiation stress (12, 25). The present data reveal that SSB levels in *D. radiodurans* follow a similar kinetics and are induced by irradiation in a time-dependent (Fig. 1a) and dose-dependent (Fig. 2a) manner. Such enhanced SSB levels during PIR are attained by transcriptional activation of the *ssb* gene. This is borne out by the facts that (i) addition of the bacterial transcription inhibitor rifampin before commencement of PIR blocks such enhancement, (ii) cells irradiated in the presence of rifampin but recovered after washing off of rifampin show enhanced SSB levels, and (iii) irradiation does not inactivate rifampin and addition of irradi-

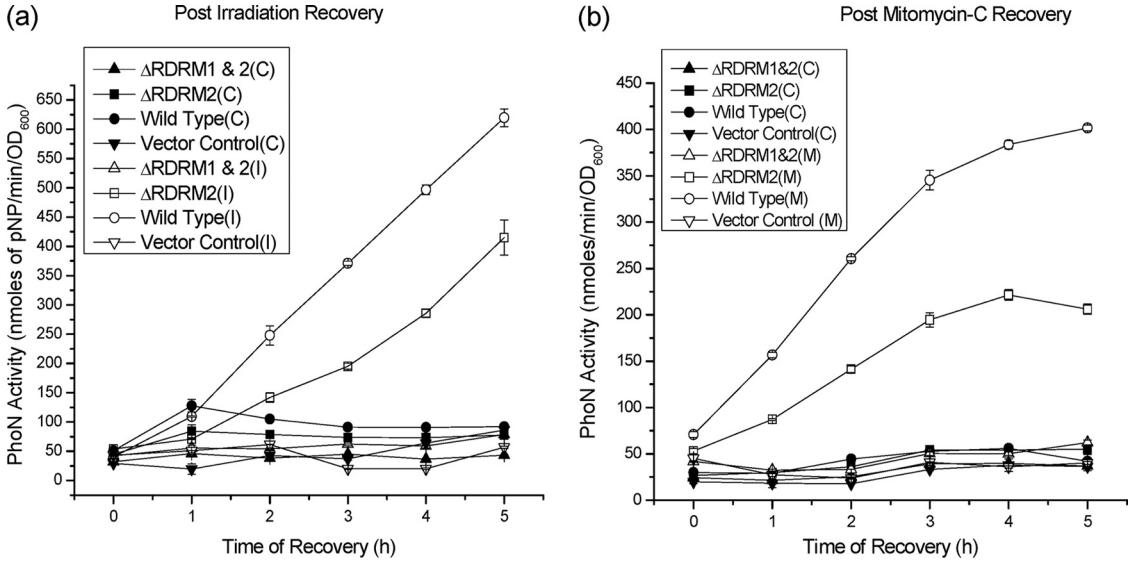

FIG. 5. Biochemical assays of PhoN reporter activity expressed from the deinococcal *ssb* promoter during poststress recovery. Recombinant *D. radiodurans* strains carrying plasmid pSN2 (ΔRDRM1 and ΔRDRM2), pSN3 (ΔRDRM2), pSN4 (wild type), or pRN1 (vector control) were exposed to either 7 kGy of ^{60}Co gamma rays (a) or 20 μg ml^{-1} mitomycin C for 15 min (b). After the stress, cells were washed and allowed to recover in TGY for 5 h. Aliquots of 100 μl were removed periodically for cell-based acid phosphatase assays using pNPP as the substrate. PhoN activity in untreated control (C), irradiated (I), or mitomycin C-treated (M) cells during recovery is shown.

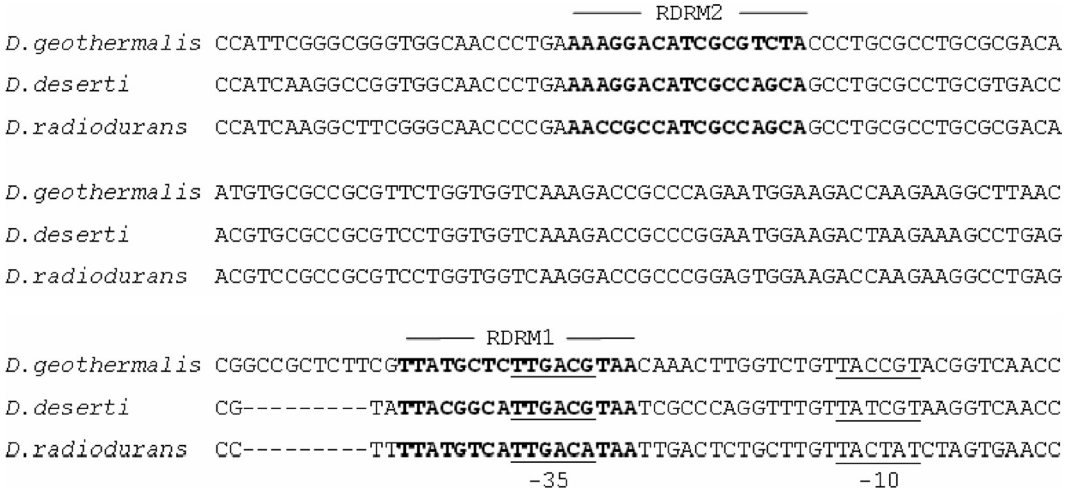

FIG. 6. Comparative bioinformatic analysis of the *ssb* promoter sequences of three *Deinococcus* spp. Nucleotide sequences of the *ssb* promoter regions of three *Deinococcus* species were aligned using ClustalW software. RDRM1 and RDRM2 sequences are shown in bold. The putative −10 and −35 sequences are underlined.

ated rifampin prior to PIR also prevents SSB upregulation during PIR (Fig. 2b).

The SSB levels in *D. radiodurans* are enhanced not only by a high dose (7 kGy) of gamma irradiation (Fig. 1a and 2a) but also by mitomycin C treatment (Fig. 1b), both of which result in abundant double-strand breaks (DSBs) in DNA or double-stranded DNA (dsDNA) adducts. UV exposure and H_2O_2 treatment, which do not produce detectable DSBs in *D. radiodurans* (27) but instead cause more pyrimidine dimers, individual base damage, or single-strand breaks, do not affect SSB expression (Fig. 1c and d). These results suggest that multiple DSBs in DNA may possibly act as the trigger for the observed response. However, prolonged desiccation, which is known to cause DNA double-strand breaks in *D. radiodurans* (27), does not enhance cellular SSB levels (Fig. 1e). This is surprising in view of the predicted commonality of response shared by *D. radiodurans* cells exposed to gamma rays or desiccation (27, 35). The underlying reasons are not clear, but a possible explanation may be that while prolonged desiccation, like radiation, does cause DSBs in DNA in *D. radiodurans*, the magnitudes of damage caused by the two stresses are very different. For example, 1 to 4 weeks of desiccation causes much less DNA damage (27) than 3 to 5 kGy of gamma irradiation (4, 27), and DNA DSBs comparable to those after a 5-kGy or higher dose of irradiation are seen only after 6 weeks of desiccation (27). It is possible, therefore, that a relatively high threshold level of DSBs is required for induction of *ssb* expression, as is also indicated by the data presented in Fig. 2a. Acute irradiation (>3 kGy) may easily exceed this threshold, while chronic slow-acting desiccation may not. An interesting related question is about how the DNA damage caused by desiccation is managed without SSB. *D. radiodurans* possesses another functional SSB-like protein, DdrB (34, 40). *ddrB* expression is enhanced in response to both irradiation and desiccation (38). This "alternate SSB" may effectively substitute for SSB function under desiccation.

The *cis* elements involved in the radiation/mitomycin-induced transcriptional activation of the *D. radiodurans ssb* gene have been elucidated. Both the RDRM1 and RDRM2 sequences appear to be important and to function in an additive manner in clone pSN4, since deletion of one (pSN3) or both (pSN2) elements progressively inhibits the P*ssb* promoter activity, as assessed in several different ways (Fig. 4 and 5). The two RDRM-like sequences of *D. radiodurans* have little in common; i.e., only eight bases, at the 5th, 7th, 8th, 9th, 11th, 13th, 14th, and 17th positions, are shared (Fig. 6). The ΔG values for RDRM1 and RDRM2 (predicted using BPROM software) at 32°C, the optimal growth temperature for *D. radiodurans*, and their GC contents are also very different. RDRM2 is very dissimilar from the predicted global RDRM (26). However, it has much higher GC content (64.7%) than RDRM1 (23.5%) and superior ΔG values (−32.4 kcal, versus −22.5 kcal for RDRM1), and it does function as a regulatory palindrome in *D. radiodurans*. Our data demonstrate that instead of simple sequence homology, the use of additional parameters such as ΔG values may aid in recognition of additional/similar regulatory palindromic sequences in bacterial genomes. Palindromes have been shown to act as *cis* elements upregulating transcription of certain eukaryotic genes (3). Recent reports based on comparative genomics have also identified certain palindromic sequences in prokaryotes which act as recognition sites for RNA polymerase or transcription factors and enhance transcription (41). More importantly, both RDRM1 and RDRM2 sequences are found at identical locations (based on ClustalW analysis [http://www.ebi.ac.uk/Tools/clustalw2/index.html]), upstream of the *ssb* ORF in all three deinococcal species (*D. radiodurans*, *D. geothermalis*, and *D. deserti*) (Fig. 6), with comparable ΔG values of −22.5 to −27.6 kcal for RDRM1 and −28.3 to −32.5 kcal for RDRM2. Our results demonstrate, for the first time, that these motifs indeed regulate radiation/mitomycin C-induced *ssb* gene expression in *D. radiodurans*. The regulatory mechanisms involving RDRM-like *cis* elements need to be explored further. Preliminary attempts in our laboratory failed to detect and isolate a *trans*-acting protein capable of binding the 351-bp-long *ssb* promoter DNA, carrying both RDRM like sequences.

Comparative genomics had very accurately predicted the possible involvement of RDRM-like sequences in radiation/desiccation-responsive expression of several genes in the three deinococcal species studied so far (6, 14, 26), though such a role had never been experimentally verified. The present work has revealed the importance and involvement of RDRM-like sequences in radiation-induced *ssb* gene expression and its insensitivity to desiccation in *D. radiodurans.* In the future, if this feature is found to be common to other genes/operons which harbor upstream RDRM-like sequences, it may be more prudent to rename this regulatory element the radiation response motif (RRM), instead of using the name RDRM given earlier.

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REFERENCES

1. **Anderson, A. W., H. C. Nordan, R. F. Cain, G. Parrish, and D. Duggan.** 1956. Studies on a radio-resistant micrococcus. I. Isolation, morphology, cultural characteristics, and resistance to gamma radiation. Food Technol. **10:**575–578.
2. **Appukuttan, D., A. S. Rao, and S. K. Apte.** 2006. Engineering of *Deinococcus radiodurans* R1 for bioprecipitation of uranium from dilute nuclear waste. Appl. Environ. Microbiol. **72:**7873–7878.
3. **Barron, E. A., Z. Cao, B. G. Schneider, E. Kraig, A. J. Carrillo, and Z. D. Sharp.** 1989. Dual functions of a *cis*-acting element within the rat prolactin gene promoter. Mol. Cell. Biol. **9:**817–819.
4. **Battista, J. R.** 1997. Against all odds: the survival strategies of *Deinococcus radiodurans*. Annu. Rev. Microbiol. **51:**203–224.
5. **Battista, J. R., M. J. Park, and A. E. McLemore.** 2001. Inactivation of two homologues of proteins presumed to be involved in the desiccation tolerance of plants sensitizes *Deinococcus radiodurans* R1 to desiccation. Cryobiology **43:**133–139.
6. **Blasius, M., S. Sommer, and U. Hubscher.** 2008. *Deinococcus radiodurans*: what belongs to the survival kit? Crit. Rev. Biochem. Mol. Biol. **43:**221–238.
7. **Bolton, P. G., and A. C. Dean.** 1972. Phosphatase synthesis in *Klebsiella* (*Aerobacter*) *aerogenes* growing in continuous culture. Biochem. J. **127:**87–96.
8. **Cox, M. M., and J. R. Battista.** 2005. *Deinococcus radiodurans*—the consummate survivor. Nat. Rev. Microbiol. **3:**882–892.
9. **Daly, M. J.** 2006. Modulating radiation resistance: insights based on defenses against reactive oxygen species in the radioresistant bacterium *Deinococcus radiodurans*. Clin. Lab. Med. **26:**491–504.
10. **Daly, M. J., E. K. Gaidamakova, V. Y. Matrosova, A. Vasilenko, M. Zhai, R. D. Leapman, B. Lai, B. Ravel, S. M. Li, K. M. Kemner, and J. K. Fredrickson.** 2007. Protein oxidation implicated as the primary determinant of bacterial radioresistance. PLoS Biol. **5:**e92.
11. **Daly, M. J., E. K. Gaidamakova, V. Y. Matrosova, A. Vasilenko, M. Zhai, A. Venkateswaran, M. Hess, M. V. Omelchenko, H. M. Kostandarithes, K. S. Makarova, L. P. Wackett, J. K. Fredrickson, and D. Ghosal.** 2004. Accumulation of Mn(II) in *Deinococcus radiodurans* facilitates gamma-radiation resistance. Science **306:**1025–1028.
12. **Daly, M. J., and K. W. Minton.** 1996. An alternative pathway of recombination of chromosomal fragments precedes *recA*-dependent recombination in the radioresistant bacterium *Deinococcus radiodurans*. J. Bacteriol. **178:**4461–4471.
13. **de Groot, A., V. Chapon, P. Servant, R. Christen, M. F. Saux, S. Sommer, and T. Heulin.** 2005. *Deinococcus deserti* sp. nov., a gamma-radiation-tolerant bacterium isolated from the Sahara Desert. Int. J. Syst. Evol. Microbiol. **55:**2441–2446.
14. **de Groot, A., R. Dulermo, P. Ortet, L. Blanchard, P. Guerin, B. Fernandez, B. Vacherie, C. Dossat, E. Jolivet, P. Siguier, M. Chandler, M. Barakat, A. Dedieu, V. Barbe, T. Heulin, S. Sommer, W. Achouak, and J. Armengaud.** 2009. Alliance of proteomics and genomics to unravel the specificities of Sahara bacterium *Deinococcus deserti*. PLoS Genet. **5:**e1000434.
15. **Earl, A. M., M. M. Mohundro, I. S. Mian, and J. R. Battista.** 2002. The IrrE protein of *Deinococcus radiodurans* R1 is a novel regulator of *recA* expression. J. Bacteriol. **184:**6216–6224.
16. **Eggington, J. M., N. Haruta, E. A. Wood, and M. M. Cox.** 2004. The single-stranded DNA-binding protein of *Deinococcus radiodurans*. BMC Microbiol. **4:**2.
17. **Ferreira, A. C., M. F. Nobre, F. A. Rainey, M. T. Silva, R. Wait, J. Burghardt, A. P. Chung, and M. S. da Costa.** 1997. *Deinococcus geothermalis* sp. nov. and *Deinococcus murrayi* sp. nov., two extremely radiation-resistant and slightly thermophilic species from hot springs. Int. J. Syst. Bacteriol. **47:**939–947.
18. **Gao, G., D. Le, L. Huang, H. Lu, I. Narumi, and Y. Hua.** 2006. Internal promoter characterization and expression of the *Deinococcus radiodurans pprI-folP* gene cluster. FEMS Microbiol. Lett. **257:**195–201.
19. **Grimsley, J. K., C. I. Masters, E. P. Clark, and K. W. Minton.** 1991. Analysis by pulsed-field gel electrophoresis of DNA double-strand breakage and repair in *Deinococcus radiodurans* and a radiosensitive mutant. Int. J. Radiat Biol. **60:**613–626.
20. **Harris, D. R., M. Tanaka, S. V. Saveliev, E. Jolivet, A. M. Earl, M. M. Cox, and J. R. Battista.** 2004. Preserving genome integrity: the DdrA protein of *Deinococcus radiodurans* R1. PLoS Biol. **2:**e304.
21. **Hua, Y., I. Narumi, G. Gao, B. Tian, K. Satoh, S. Kitayama, and B. Shen.** 2003. PprI: a general switch responsible for extreme radioresistance of *Deinococcus radiodurans*. Biochem. Biophys. Res. Commun. **306:**354–360.
22. **Joshi, B., R. Schmid, K. Altendorf, and S. K. Apte.** 2004. Protein recycling is a major component of post-irradiation recovery in *Deinococcus radiodurans* strain R1. Biochem. Biophys. Res. Commun. **320:**1112–1117.
23. **Levin-Zaidman, S., J. Englander, E. Shimoni, A. K. Sharma, K. W. Minton, and A. Minsky.** 2003. Ringlike structure of the *Deinococcus radiodurans* genome: a key to radioresistance? Science **299:**254–256.
24. **Lipton, M. S., L. Pasa-Tolic, G. A. Anderson, D. J. Anderson, D. L. Auberry, J. R. Battista, M. J. Daly, J. Fredrickson, K. K. Hixson, H. Kostandarithes, C. Masselon, L. M. Markillie, R. J. Moore, M. F. Romine, Y. Shen, E. Stritmatter, N. Tolic, H. R. Udseth, A. Venkateswaran, K. K. Wong, R. Zhao, and R. D. Smith.** 2002. Global analysis of the *Deinococcus radiodurans* proteome by using accurate mass tags. Proc. Natl. Acad. Sci. U. S. A. **99:**11049–11054.
25. **Liu, Y., J. Zhou, M. V. Omelchenko, A. S. Beliaev, A. Venkateswaran, J. Stair, L. Wu, D. K. Thompson, D. Xu, I. B. Rogozin, E. K. Gaidamakova, M. Zhai, K. S. Makarova, E. V. Koonin, and M. J. Daly.** 2003. Transcriptome dynamics of *Deinococcus radiodurans* recovering from ionizing radiation. Proc. Natl. Acad. Sci. U. S. A. **100:**4191–4196.
26. **Makarova, K. S., M. V. Omelchenko, E. K. Gaidamakova, V. Y. Matrosova, A. Vasilenko, M. Zhai, A. Lapidus, A. Copeland, E. Kim, M. Land, K. Mavrommatis, S. Pitluck, P. M. Richardson, C. Detter, T. Brettin, E. Saunders, B. Lai, B. Ravel, K. M. Kemner, Y. I. Wolf, A. Sorokin, A. V. Gerasimova, M. S. Gelfand, J. K. Fredrickson, E. V. Koonin, and M. J. Daly.** 2007. *Deinococcus geothermalis*: the pool of extreme radiation resistance genes shrinks. PLoS One **2:**e955.
27. **Mattimore, V., and J. R. Battista.** 1996. Radioresistance of *Deinococcus radiodurans*: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. J. Bacteriol. **178:**633–637.
28. **Meima, R., and M. E. Lidstrom.** 2000. Characterization of the minimal replicon of a cryptic *Deinococcus radiodurans* SARK plasmid and development of versatile *Escherichia coli-D. radiodurans* shuttle vectors. Appl. Environ. Microbiol. **66:**3856–3867.
29. **Meima, R., H. M. Rothfuss, L. Gewin, and M. E. Lidstrom.** 2001. Promoter cloning in the radioresistant bacterium *Deinococcus radiodurans*. J. Bacteriol. **183:**3169–3175.
30. **Minton, K. W.** 1994. DNA repair in the extremely radioresistant bacterium *Deinococcus radiodurans*. Mol. Microbiol. **13:**9–15.
31. **Moseley, B. E., and A. Mattingly.** 1971. Repair of irradiation transforming deoxyribonucleic acid in wild type and a radiation-sensitive mutant of *Micrococcus radiodurans*. J. Bacteriol. **105:**976–983.
32. **Narumi, I., K. Satoh, S. Cui, T. Funayama, S. Kitayama, and H. Watanabe.** 2004. PprA: a novel protein from *Deinococcus radiodurans* that stimulates DNA ligation. Mol. Microbiol. **54:**278–285.
33. **Narumi, I., K. Satoh, M. Kikuchi, T. Funayama, T. Yanagisawa, Y. Kobayashi, H. Watanabe, and K. Yamamoto.** 2001. The LexA protein from *Deinococcus radiodurans* is not involved in RecA induction following gamma irradiation. J. Bacteriol. **183:**6951–6956.
34. **Norais, C. A., S. Chitteni-Pattu, E. A. Wood, R. B. Inman, and M. M. Cox.** 2009. DdrB protein, an alternative *Deinococcus radiodurans* SSB induced by ionizing radiation. J. Biol. Chem. **284:**21402–21411.
35. **Rainey, F. A., M. Ferreira, M. F. Nobre, K. Ray, D. Bagaley, A. M. Earl, J. R. Battista, B. Gomez-Silva, C. P. McKay, and M. S. da Costa.** 2007. *Deinococcus peraridilitoris* sp. nov., isolated from a coastal desert. Int. J. Syst. Evol. Microbiol. **57:**1408–1412.
36. **Riccio, M. L., G. M. Rossolini, G. Lombardi, A. Chiesurin, and G. Satta.** 1997. Expression cloning of different bacterial phosphatase-encoding genes by histochemical screening of genomic libraries onto an indicator medium containing phenolphthalein diphosphate and methyl green. J. Appl. Microbiol. **82:**177–185.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
38. **Tanaka, M., A. M. Earl, H. A. Howell, M. J. Park, J. A. Eisen, S. N. Peterson, and J. R. Battista.** 2004. Analysis of *Deinococcus radiodurans*'s transcriptional response to ionizing radiation and desiccation reveals novel proteins that contribute to extreme radioresistance. Genetics **168:**21–33.
39. **White, O., J. A. Eisen, J. F. Heidelberg, E. K. Hickey, J. D. Peterson, R. J.**

Dodson, D. H. Haft, M. L. Gwinn, W. C. Nelson, D. L. Richardson, K. S. Moffat, H. Qin, L. Jiang, W. Pamphile, M. Crosby, M. Shen, J. J. Vamathevan, P. Lam, L. McDonald, T. Utterback, C. Zalewski, K. S. Makarova, L. Aravind, M. J. Daly, K. W. Minton, R. D. Fleischmann, K. A. Ketchum, K. E. Nelson, S. Salzberg, H. O. Smith, J. C. Venter, and C. M. Fraser. 1999. Genome sequence of the radioresistant bacterium *Deinococcus radiodurans* R1. Science **286:**1571–1577.

40. **Xu, G., H. Lu, L. Wang, H. Chen, Z. Xu, Y. Hu, B. Tian, and Y. Hua.** 2010. DdrB stimulates single-stranded DNA annealing and facilitates RecA-independent DNA repair in *Deinococcus radiodurans*. DNA Repair **9:**805–812.
41. **Yannone, S. M., and B. K. Burgess.** 1997. Identification of a palindromic sequence that is responsible for the up-regulation of NAPDH-ferredoxin reductase in a ferredoxin I deletion strain of *Azotobacter vinelandii*. J. Biol. Chem. **272:**14454–14458.
42. **Zahradka, K., D. Slade, A. Bailone, S. Sommer, D. Averbeck, M. Petranovic, A. B. Lindner, and M. Radman.** 2006. Reassembly of shattered chromosomes in *Deinococcus radiodurans*. Nature **443:**569–573.
43. **Zimmerman, J. M., and J. R. Battista.** 2005. A ring-like nucleoid is not necessary for radioresistance in the *Deinococcaceae*. BMC Microbiol. **5:**17.