REGULATION AND FUNCTION OF MULTIPLE *SSB* GENES OF *ANABAENA* PCC7120

By

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Anurag Kirti

DECLARATION

I, hereby declare that the investigation presented in this 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.

Anurag Kirti

Dedicated to

My family and friends

Acknowledgement

This thesis is the culmination of my Ph.D work and is seeing the day due to support and encouragement of numerous people including my well wishers, my friends and colleagues. And I take pleasure in expressing my thanks to all those who have contributed in many ways to the success of this study.

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Anurag Kirti

TABLE OF CONTENTS

Synopsis

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SYNOPSIS

(Limited to 10 pages in double spacing)

Cyanobacteria (blue-green algae) are ancient photosynthetic organisms and considered as progenitors of chloroplasts present in plant cells (1, 2). They have been exposed to and survived a variety of environmental stresses, such as heat, drought, salinity, photo-oxidation, ultraviolet and ionizing radiation stresses (3, 4). The filamentous heterocystous nitrogen-fixing cyanobacterium, *Anabaena* sp. strain PCC7120, whose complete genome sequence is known (5), is a highly γ-radiation (LD_{50}= 6 kGy, D_{10}= 12 kGy) and desiccation tolerant organism (6, 7). In the highly radioresistant bacterium, *Deinococcus*, the high radioresistance has been related to its efficient DNA repair system (8). Generation of intact DNA from the shattered chromosomal DNA during post-irradiation recovery has also been observed for *Anabaena* 7120 (7). However, not much is known about the mechanism of DNA repair in cyanobacteria.

During DNA repair, recombination or replication, the DNA double strand needs to be transiently unwound and the single-stranded DNA (ssDNA), thus generated needs to be protected from digestion by nucleases. As the duplex DNA is unwound, single-stranded DNA binding proteins (SSBs) bind and stabilize ssDNA conformation until it is utilized by downstream proteins involved in the corresponding pathways (9). SSBs bind ssDNA non-specifically through a conserved structural motif called OB-fold (oligonucleotide/oligosaccharide binding fold) present at the N-terminus, through a combination of electrostatic, hydrogen-bonding and stacking interactions (10). The OB-fold of SSB is immediately followed by a proline/glycine (P/G)-rich spacer region, separating the positively charged OB-fold from the less conserved and highly negatively charged C-terminal (acidic) tail responsible for protein-protein interaction (11). SSBs interact with a number of proteins involved in establishment and maintenance of genome, which include RecA, DNA polymerase, primase, uracil DNA glycosylase (UDG), DNA polymerase II (Pol II). SSBs not only allow targeting of these proteins at repair site, but also influence their activity (9).

In general, SSB proteins are known to function as oligomers. For example in *E. coli* these function as tetramer (12), while in *Deinococcus*–thermus genera form dimers (13). Trimeric and monomeric forms of SSB have also been reported in humans and bacteriophages respectively (14, 15). The existence of dimeric and tetrameric forms of SSB dictate the binding modes of SSB i.e. the number of interacting OB-folds with ssDNA which is two OB-folds for a dimer that covers about 35 bases, and four OB-folds for a tetramer that covers about 65 bases (16). The generation of these binding modes is dependent on salt concentration as well as protein to ssDNA ratio. The different binding modes may be used selectively during replication, recombination or repair *in vivo*, allowing non-specific recognition (17). SSB is an essential protein in bacteria. Its gene mutation impairs DNA

repair and replication and is lethal (12). On the other hand, overexpression of SSB has a mixed response, enhancing base excision repair, but decreasing recombinational repair (18).

In general, most bacteria possess a single *ssb* gene. However, a few bacteria such as *Bacillus subtilis* have two SSBs, each having a specific function (19). Multiple SSBs have also been reported in cyanobacteria (http://genome.microbedb.jp/cyanobase). Expression of SSB is generally regulated by the repressor LexA which binds the SOS-Box (20). The other modes of regulation, which include cis-acting RDRM element in *Deinococcus* (21) and negative regulation of its translation by binding to its own mRNA have also been reported (22).

Objectives:

- Cloning of three *ssb* genes of *Anabaena sp*. PCC7120 in *E. coli* for overexpression.
- Purification of these SSB proteins, their biochemical characterization and preparation of polyclonal antibody for each of them.
- Profiling SSB protein expression under different stress conditions in Anabaena.
- Modulation of individual SSB levels in Anabaena by overexpression/site-directed mutation in each of the genes and assessment of its physiological significance.
- Transcriptional analysis of the *ssb* genes to ascertain whether it is monocistronic or polycistronic.
- Elucidation of regulatory mechanisms involved in *ssb* gene expression.

Organization of thesis:

The thesis is divided into six chapters. These include introduction and review of literature (Chapter 1), materials and methods (Chapter 2) and three chapters (3, 4 and 5) describing the experimental results including the relevant discussion. A summary (Chapter 6) of the major findings from this work is presented at the end, followed by references of the literature cited.

Chapter 1: Introduction

The first chapter provides a literature review on structural and biochemical properties, and physiological role of the SSB proteins in bacteria, with major emphasis on *E. coli*. A brief outline on different DNA-damage inducing stresses and their subsequent repair has been given. A general overview on cyanobacteria, covering its response to different DNA-damaging stresses have also been discussed. The chapter concludes with a paragraph on the outline of thesis work on the three SSB-like proteins (Alr0088, Alr7579 and All4779) of *Anabaena* 7120.

Chapter 2: Materials and Methods

The second chapter lists different bacterial strains and various experimental techniques used in the present study. The different plasmids used/generated during the course of study, the primers used for PCR amplification and the reagents used for different experimental procedures have been tabulated. The procedures followed for isolation of plasmid and chromosomal DNA, RNA, PCR amplification including Reverse-Transcriptase (RT)-PCR and cloning of DNA fragments into appropriate cloning/expression vectors have been described in detail. The major protocols followed for overexpression, purification, electrophoretic separation and visualisation of proteins have been described. Biochemical assays which included size determination by HPLC-based gel filtration and glutaraldehyde cross-linking, and interaction with ssDNA by Fluorescence Quenching technique and Electro-Mobility Shift Assay (EMSA) has also been described. Generation of recombinant *Anabaena* strains harbouring different stable plasmid constructs, assessment of promoter activity using reporter gene assay and contribution of individual genes to stress tolerance of *Anabaena* have also been detailed.

Chapter 3: Biochemical characterization of *Anabaena* SSB proteins

A brief introduction is followed by *in silico* analysis of the multiple *ssb*-like genes of *Anabaena* 7120 and comparison with the corresponding proteins of different bacteria. In cyanobacteria, two ORFs annotated as SSB, *alr0088* and *alr7579* have been designated as SSB1 and SSB2 respectively, while another ORF annotated as hypothetical protein but possessing structural motif typical of SSB proteins, was designated as SSB3. SSB1 and SSB2 are 119 and 127 amino acids long, coding for 13.1 kDa and 14 kDa proteins respectively, but having only the N-terminal OB-fold. The 182 amino acid long, 20 kDa SSB3, on the other hand, has the P/G-rich region as well as acidic tail downstream to the N-terminal OB, typical of most bacterial SSBs. The corresponding genes were individually PCR amplified from *Anabaena* 7120 and cloned into expression vector pET16b. The corresponding proteins were overexpressed in *E. coli* upon induction with IPTG and purified by Ni-NTA affinity chromatography to near homogeneity. The different steps involved in the above procedure have been described in detail in this chapter. Gel filtration and glutaraldehyde based cross-linking indicated that all three SSB proteins existed primarily either as a monomer or a dimer, while low levels of tetrameric form of SSB3 were detected in the presence of ssDNA. The interaction of the three SSB proteins with ssDNA was assayed by fluorescence quenching and EMSA techniques. Quenching of intrinsic fluorescence of SSB1 and SSB3 could be achieved with both poly(dT) and M13 ssDNA, while that of SSB2 only with native but not with heat-denatured M13 ssDNA. Based on fluorescence quenching with poly(dT), the binding affinity was calculated as $2.56 \pm 0.4 \times 10^6$ M^{-1} and $5.13 \pm 0.71 \times 10^7$ M^{-1} for SSB1 and SSB3, respectively. SSB1 exhibited a single occlusion site of 54-55 bases irrespective of salt concentration, while SSB3 showed NaCl concentration dependent two binding modes namely 35 and 66 bases, respectively at 20 mM and 100 mM NaCl. Of the three SSB proteins, SSB3 exhibited biochemical properties closest to that of *E. coli* SSB and may be the typical bacterial SSB of *Anabaena* 7120.

Chapter 4: Stress-induced regulation of *Anabaena* SSB proteins and identification of the regulatory regions of the corresponding genes

This chapter begins with *in silico* analysis of *ssb* gene organization and their putative promoter regions. The *ssb1* gene was found to be monocistronic, *ssb2* was the first gene in a series of four genes transcribed in the same direction, while *ssb3* was the first gene of a bicistronic operon. The individual promoter regions of these genes were PCR amplified and cloned in a promoterless shuttle vector, pAM1956, to assess their activity under normal conditions as well as during DNA damage inducing conditions in both *E. coli* and *Anabaena*. An ~400 bp region upstream of the translational start codon of ORF *ssb1*, *ssb2* and *ssb3* were individually amplified using $P_{(ssb1/2/3)}$Fwd and $P_{(ssb1/2/3)}$Rev specific primers. These fragments were cloned into pAM1956, wherein it could control the expression of the downstream *gfpmut2* reporter gene and introduced into *Anabaena* by conjugation. The recombinant *Anabaena* strains An(pAM-P_{ssb1}::gfp) and An(pAM-P_{ssb2}::gfp) showed green fluorescence at λ_{ex}= 489 nm and λ_{em}= 508 nm under microscope, while An(pAM-P_{ssb3}::gfp) fluoresced red indicative of only chlorophyll fluorescence. The 350 bp region upstream of *ssb3* has a large AT-rich track which could be interfering with the functioning of the promoter. Thus, a 150 bp region upstream of *ssb3* devoid of the AT-rich track was cloned and the recombinant *Anabaena* strain An(pAM-P_{ssb3s}::gfp) was generated which exhibited green fluorescence. The activities of all these promoters were assessed fluorimetrically under normal growth conditions and during different DNA-damage stresses. Based on these results, it was concluded that the promoters of *ssb1* and *ssb2* genes are active mainly during the logarithmic phase (3-4 days) and activity decreases during stationary phase (beyond 5 days). On the other hand, *ssb3s* promoter activity increased up to day 7. Exposure to mitomycin C did not significantly affect activity of *ssb1*, *ssb2* and *ssb3s* promoters. Exposure to γ-irradiation and post-irradiation recovery increased activity of

ssb2 and *ssb3s* promoters, but decreased that of *ssb1* promoter, while post desiccation recovery lead to increase in *ssb1* and *ssb3*s promoter activity. The stress-regulated changes in the activity of the promoter were reflected on the changes at protein level for each of the three SSB proteins. Exposure to mitomycin C or UV-B irradiation only marginally changed the levels of the SSB proteins, and this change was dose-dependent, except for a significant increase observed in the levels of SSB3 upon exposure to UV-B. The expression of both SSB2 and SSB3 were enhanced during post-irradiation recovery, while desiccation stress enhanced the expression of SSB2 while decreasing that of SSB3. Expression of SSB1 was unaffected by either irradiation or desiccation stress. EMSA studies in the presence of *Anabaena* LexA revealed a shift in the mobility of the *ssb1* and *ssb2* promoters, both of which contain a putative LexA-Box, but not *ssb3* promoter, which does not have LexA box.

Chapter 5: Possible physiological roles of SSB proteins

This chapter deals with the generation of recombinant *Anabaena* strains with either mutation of one of the *ssb* genes or overexpressing one of the SSB proteins and analysis of their *in vivo* role. Insertional mutagenesis of each *ssb* gene individually with *nptII* cassette in *Anabaena* was lethal suggesting that each of the *ssb* genes may be having a distinct role and hence essential. On the other hand, overexpression of the individual SSB proteins did not affect normal cell physiology of *Anabaena*. Overexpression was achieved from a light-inducible promoter P_{psbA1} which also co-expressed Green Fluorescent Protein (GFP) transcribed from the transcriptionally fused downstream *gfpmutII* gene. The overexpression of the corresponding SSB proteins in the recombinant *Anabaena* strains (An*ssb1*$^{+}$, An*ssb2*$^{+}$ and An*ssb3*$^{+}$) was also confirmed by western blotting and immunodetection with their respective antibodies. These strains were tested for their tolerance towards DNA damage inducing stresses as compared to empty vector control, AnpAM. Overexpression of SSB1 increased tolerance towards mitomycin C and UV-B stress, but decreased

tolerance to γ-irradiation and desiccation stress, while overexpression of SSB2 had no significant effect on tolerance of *Anabaena* to any of the stresses tested. On the other hand, overexpression of SSB3 enhanced tolerance to agents causing DNA adduct formation (UV-B and Mitomycin C) as well as those causing ss and ds breaks (γ-irradiation and desiccation). Thus, SSB3 may be the major SSB of *Anabaena* involved in DNA repair.

Chapter 6: Summary:

In the genome of *Anabaena* sp. strain PCC 7120, two genes annotated as *alr0088* and *alr7579* designated as *ssb1* and *ssb2*, respectively coding for proteins having only N-terminal OB-fold and no P/G rich region or acidic tail. However, another ORF *'all4779'*, annotated as a hypothetical protein, has an N-terminal OB-fold, a P/G-rich region and a C-terminal acidic tail, typical of SSB. All these proteins were overexpressed in *E. coli* and purified under native conditions. Unlike major bacterial SSBs, the *Anabaena* SSBs did not form a tetramer, and existed as dimers except for SSB3 which formed tetramer in presence of ssDNA. Due to the presence of the OB-fold, all three SSB proteins bound ssDNA, but the binding was dependent on type of ssDNA (poly dT or M13 ssDNA) and NaCl concentration. Of these three proteins, SSB3 exhibited maximum binding affinity, almost equivalent to that of *E. coli* SSB. SSB1 exhibited 10-fold lower binding affinity in spite of the absence of the C-terminal tail which otherwise is known to decrease the binding affinity. SSB2 on the other hand, bound only native M13 ssDNA and not heat denatured M13 ssDNA or poly (dT), suggesting that it might be recognizing secondary structures. The SSB1 bound ssDNA in a single binding mode, while SSB3 exhibited two binding modes similar to *E. coli* SSB. Thus, in all respects the *Anabaena* SSB3 may be the typical bacterial SSB. A differential expression of the three SSB proteins in response to DNA damaging stresses was observed, regulated at transcriptional level.

The expression of *ssb1* was negatively regulated by LexA of *Anabaena* and *ssb3* by an AT-rich element. Mutation of even one of the *ssb* genes was lethal, indicating critical function for each of them. SSB1 overexpression enhanced tolerance to agents causing DNA adducts formation, but decreased to those causing single and double strand breaks. Due to the absence of the acidic tail in SSB1, the localisation of repair proteins to the site of repair would not be possible, thus hampering repair. Thus, the main function of SSB1 could be to protect DNA from damage rather than repair *per se*. While, the overexpression had no effect on stress tolerance and could be due to its weak interaction with ssDNA. SSB3, on the other hand, enhanced protection against all the DNA damage inducing agents tested. Possibly the presence of the acidic tail in SSB3 allowed interaction with DNA repair proteins. Thus, it is proposed that 'All4779' (SSB3) be re-annotated a SSB in the genome database.

References:

1. Brock TD (1973) Science 179: 480-483.
2. Schopf JW (1975) Ann. Rev. Earth Planetary Sci. 3: 213-249.
3. Fay P (1992) Microbiol. Rev. 56: 340-373.
4. Apte SK (2001) Proc. Indian Natl. Sci. Acad. 5: 285-310.
5. Kaneko T, Nakamura Y, Wolk CP, Kuritz T, Sasamoto S et al. (2001) DNA Res. 8: 205-213; 227-253.
6. Singh H, Fernandes T, Apte SK (2010) J. Biosci. 35: 427-434.
7. Singh H, Anurag K, Apte SK (2013) Photosynth. Res. 118:71-81
8. Battista JR (1997) Annu. Rev. Microbiol. 51: 203-224.
9. Shereda RD, Kozlov AG, Lohman TM, Cox MM, Keck JL. (2008) Crit Rev Biochem Mol Biol. 43: 289-318.

10. Lohman TM, Ferrari ME (1994) Annu. Rev. Biochem. 63: 527-570.

11. Lu D, Keck JL (2008) Proc. Natl. Acad. Sci. U S A. 105: 9169-9174.

12. Meyer RR and Laine PS (1990) Microbiol. Rev. 54: 342-380.

13. Eggington JM, Haruta N, Wood EA, Cox MM. (2004) BMC Microbiol. 4:2

14. Wold MS (1997) Annu Rev Biochem. 66:61-92.

15. Shamoo Y, Friedman AM, Parsons MR, Konigsberg WH, Steitz TA. (1995) Nature. 376: 362-366.

16. Bujalowski W, Lohman TM. (1986) Biochem. 25: 7799-7802

17. Kozlov AG, Lohman TM. (2002) Biochem. 41:11611-11627

18. Moreau PL (1987). J Mol Biol. 194: 621-634

19. Yadav T, Carrasco B, Myers AR, George NP, Keck JL, Alonso JC (2012) Nucleic Acids Res. 40: 5546-5559.

20. Perrino, FW, Rein DC, Bobst AM, and Meyer RR. (1987) Mol. Gen. Genet. 209: 612-614.

21. Ujaoney AK, Potnis AA, Kane P, Mukhopadhyaya R, Apte SK (2010) J Bacteriol. 192: 5637-5644.

22. Shimamoto N, Ikushima N, Utiyama H, Tachibana H, Horie K (1987) Nucleic Acids Res. 15: 5241-5250.

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2. Kirti A, Rajaram H, Apte S.K. (2012) The hypothetical protein 'All4779' of *Anabaena* sp. strain PCC7120 encodes a single stranded DNA binding protein. In: 36th All India Cell Biology Conference and International Symposium on 'Stress Response and genome Integrity (SARGI)' at BARC, Mumbai (Oct 17-19, 2012). D2RC2R8, p131
3. Kirti A*, Rajaram H, Apte S.K. (2012) Characterisation of three single-stranded DNA binding (SSB)-like proteins from the nitrogen-fixing cyanobacterium *Anabaena* PCC7120.

In: Indo-US Workshop on Cyanobacteria: Molecular Networks to Biofuels' at Lonavala, India (Dec 16-20, 2012). P1, p35

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4.	Dr. S. K. Apte	Member		
5.	Dr. Vinay Kumar	Member		
6.	Dr. H. S. Misra	Member		

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

µL	Microlitre
µM	Micromolar
bp	base pair
BPB	Bromo phenol blue
Cb	Carbenicillin
Cm	Chloramphenicol
dsDNA	Double stranded DNA
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
IPTG	Isopropyl-beta-D-thiogalactopyranoside
Kan	Kanamycin
kb	Kilo Base
kDa	Kilo Dalton
NBT/BCIP	Nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate
Ni-NTA	Nickel-Nitrilo triacetic acid
Neo	Neomycin
PAGE	Poly Acrylamide Gel Electrophoresis
PCC	Pasture Culture Collection
PCR	Polymerase Chain Reaction
PMSF	Phenyl Methyl Sulfonyl Fluoruide
SDS	Sodium dodecyl sulphate
SSB	Single stranded DNA Binding protein
ssDNA	Single stranded DNA
Tris	Tris (hydroxymethyl)-aminomethane

CHAPTER 1

Introduction

1.1 SINGLE-STRANDED DNA BINDING PROTEINS

Single-stranded (ss) DNA binding proteins are proteins which bind ssDNA non-specifically. The existence of these proteins was first discovered in the laboratory of Bruce Alberts in the late 1960's and early 1970's using DNA-cellulose affinity chromatography (1). This was first identified and characterised as a bacteriophage T4gp32 protein which bound tightly to DNA-cellulose and required almost 2 M NaCl to elute from the column indicating high affinity to DNA (1). This was followed by the identification of a similar protein from *Escherichia coli* (2, 3). This protein was initially designated as a DNA-unwinding protein from *E. coli* due to its apparent ability to destabilize and unwind a DNA double helix (1). The other names for this protein included DNA melting proteins and helix-destabilizing proteins (4), but the name which is most commonly used is single-stranded DNA binding (SSB) protein across bacteria, yeast and other organisms. These are found to be essential for DNA replication across all organisms (5). Biochemical and biophysical studies have shown that SSB's have a strong binding preference for DNA compared to RNA and specificity for ssDNA compared to duplex ds DNA.

Although DNA, an important component of life in unicellular and multicellular organisms, exists mostly in double-stranded helix form, the transfer of genetic information either during replication or synthesis of proteins, vital for the proper functioning of various cellular processes, requires unwinding of the duplex DNA. This results in the generation of single-stranded (ss) DNA intermediates, which serve as templates for these functions. However, exposure of ssDNA presents several problems to the cell due to it being thermodynamically less stable than dsDNA. This leads to

spontaneous formation of duplex secondary structures that impede genome maintenance processes. Also, the ssDNA is more sensitive to chemical and nucleolytic attacks compared to dsDNA, and this can cause damage to the genome (6). This necessitates stabilization and protection of ssDNA, which is achieved by SSBs. The existence of ssDNA, coated with SSB provides a structure recognized by a variety of enzymes involved in replication, repair and recombination (7). In addition to the above processes, generation of short ssDNA also occur when cells are exposed to certain DNA damaging agents such as UV and γ-irradiation, mitomycin C etc. as briefly discussed here:

1.2 DNA DAMAGE INDUCING AGENTS

Mitomycin C has been recognized as the first natural antibiotic isolated from *Streptomyces* cultures. It possesses monofunctional and bifunctional DNA alkylating activity causing DNA cross-links, and acting as a potent inducer of SOS response. Alkylating activity of mitomycin C generates two types of DNA adducts, 2,7-diaminomitosene (2,7-DAM) and 10-decarbamoyl mitomycin C (DMC) (8). These DNA adducts are known to activate DNA repair pathway and induce cell death in mammalian cells. Low concentration ($< 0.1 \mu g\ mL^{-1}$) is bacteriostatic while concentrations above 5 $\mu g\ mL^{-1}$ is known to be bactericidal, as it inhibits DNA synthesis completely (9). Exposure to mitomycin C has been shown to lead to the induction of SSB and RecA protein in *E. coli* (10). However, whether this induction results as a direct effect of DNA damage or as part of SOS-response has been debated (11). An *ssb* mutant (*ssb*-3) wherein Glycine-15 is replaced by Aspartate leads to high sensitivity to mitomycin C (12). Similarly, deletion of C-terminal region of RecA results in increased sensitivity to mitomycin C (10).

DNA damages by UVR result due to direct absorption of UV-B radiation by the native DNA molecule and indirectly by oxidative stress. The main DNA lesions which develop by direct UV-B absorption are dimeric photoproducts such as cis-syn cyclobutane pyrimidine dimmers (CPDs), and pyrimidine (6-4) pyrimidone photoproducts (6-4 PPs) and their Dewar isomers (13). Oxidative stress, on the other hand, generally results in single- or double-strand breaks in the native DNA molecule (14).

γ-rays induce lesions in DNA which do not directly cause strand breaks, but render them susceptible to endonucleases (15, 16). These sites are, generally, rapidly removed during and immediately after irradiation (15). The ds breaks which appear from the unrepaired single strand breaks are thought to be due to the lack of or decreased enzymatic activity, such as that of DNA Pol I (17). As a consequence of unbalanced action of repair enzymes i.e. the nucleases which enlarge the single strand gaps in DNA strands and polymerases which fill in these gaps, an increase in double strand breaks occur (18). The generation of ss breaks on DNA directly by radiation exposure is higher than that generated by conversion of enzyme labile sites to ss breaks (18). The ability to repair the ss and ds breaks determine the radioresistance of the organism e.g. *Deinococcus radiodurans* which efficiently stitches back its damaged DNA during post-irradiation recovery exhibits a high D_{10} of about 10-12 kGy (19), while *E. coli* which is unable to do so exhibits low D_{10} of only 0.1-0.5 kGy of γ-rays (20). In general, DNA has been considered as a primary radiation target. The susceptibility of *Deinococcus* to double strand break is far lower than that observed in other bacteria such as *E. coli*, which are radiation sensitive. Also, better protection of the proteome from ROS-induced

damage has been observed in *Deinococcus* compared to that in radiation sensitive bacteria (21). Thus, the level of protection from protein damage in addition to DNA damage appear to determine the radiotolerance of the organism (22). However, recent reports have indicated the importance of three DNA metabolism genes, *recA*, *dnaB* and *yfjK*, wherein single point mutations in these genes increases the radio-resistance of *E. coli* increases to 3-10 kGy (23, 24).

Desiccation also causes generation of DSBs and the damage caused in response to desiccation exhibits a significant overlap with that of gamma radiation in case *D. radiodurans* (25). Additionally, it can also cause alkylation, oxidation or depurination of DNA (26).

1.3 STRUCTURAL FEATURES OF SINGLE STRANDED DNA BINDING (SSB) PROTEINS

In spite of the ubiquitous presence of SSB, the proteins share low sequence similarity, oligomeric status and composition of subunits (7). In general SSB proteins are identified by two common structural features: (i) N-terminal domain having Oligonucleotide/oligosaccharide binding (OB) fold implicated in DNA binding through a combination of electrostatic and base-stacking interactions with the phosphodiester backbone and nucleotide bases respectively, and (ii) C-terminal domain having 8-10 amino acids long acidic tail responsible for protein-protein interaction (6).

The presence of OB-fold is, however, not specific to SSB proteins, but present in several proteins which bind ssDNA with high affinity. The proteins sharing OB-folds may show little sequence specificity. A motif comprising of five-stranded β-barrel and a

α- helix cap between the third and fourth strand is also observed in several DNA interacting proteins such as staphylococcal nuclease, certain cytotoxins etc. (27). These structures have similar topology and architecture in spite of having no significant sequence similarity. Due to the ability of such structures to bind both oligosaccharide and oligonucleotides, this motif has been designated as the OB (oligomer binding)-fold (27). The closed five-stranded β-barrel structure of the OB-fold **(Fig 1.1)** presents a binding face which allows easy adaptation for binding of wide range of biological molecules such as proteins, metal ions and catalytic substrates in addition to oligosaccharides or oligonucleotides (28). The conservation of architecture, topology and a fold related binding face suggests a divergent evolution of the OB-fold structure. However, this does not rule out its convergent evolution, as these two different modes of evolution cannot be easily differentiated owing to their stable architecture (28).

OB-folds were earlier classified in eight distinct superfamilies within the SCOP (Structural Classification of Proteins) database (28) as also shown in **Fig 1.2**. Recent advances in SCOP classification has resulted in inclusion of 16 superfamilies in SCOP classification of OB-fold proteins (http://scop.berkeley.edu/sunid=50198). The largest superfamily is the nucleic-acid binding superfamily, wherein the common ligand is a nucleic acid (28). The proteins of this superfamily are critical for DNA replication, recombination and repair, transcription, translation, cold-shock response and telomere maintenance (29). Owing to their multiple roles related to DNA damage response checkpoint, DNA repair etc, the OB-fold has been suggested to be an evolutionary conserved functional module playing a role of a genome guardian (30). The length of this domain ranges from 70 to 150 amino acids. The variability in length among OB-fold

domains is primarily due to the differences in the length of variable loops found between β-strands which form a well conserved secondary structure (29). On the basis of the kind of nucleic acid acting as ligand, the nucleic-acid binding superfamily is divided into three categories: (a) proteins that bind nucleic acids without any strong sequence specificity e.g. *E. coli* SSB (EcoSSB), Human replication protein (RPA), (b) proteins that recognize specific single-stranded regions of nucleic acids e.g. *E. coli* Rho transcriptional terminator (EcRho) and *E. coli* aspartyl-tRNA synthetase (AspRS), and (c) proteins that interact with mainly non-helical structured nucleic acids e.g. *Thermatoga maritima* RecG, ribosomal proteins and *Thermus thermophilus* initiation factor 1 (IF1) (29).

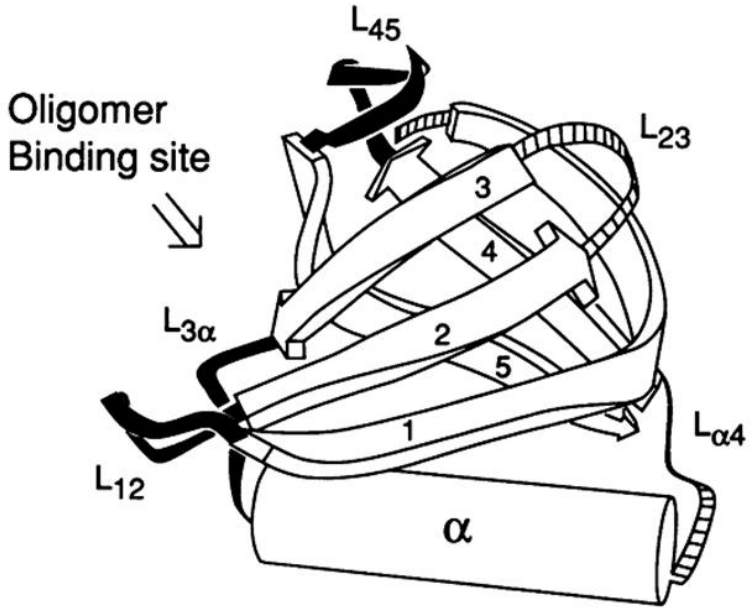

Fig. 1.1. OB-fold domain. The five β-strands are numbered 1-5 forming a closed β-barrel, capped by an α-helix cylinder. (Adapted from Murzin, 1993, EMBOJ 12:861-867)

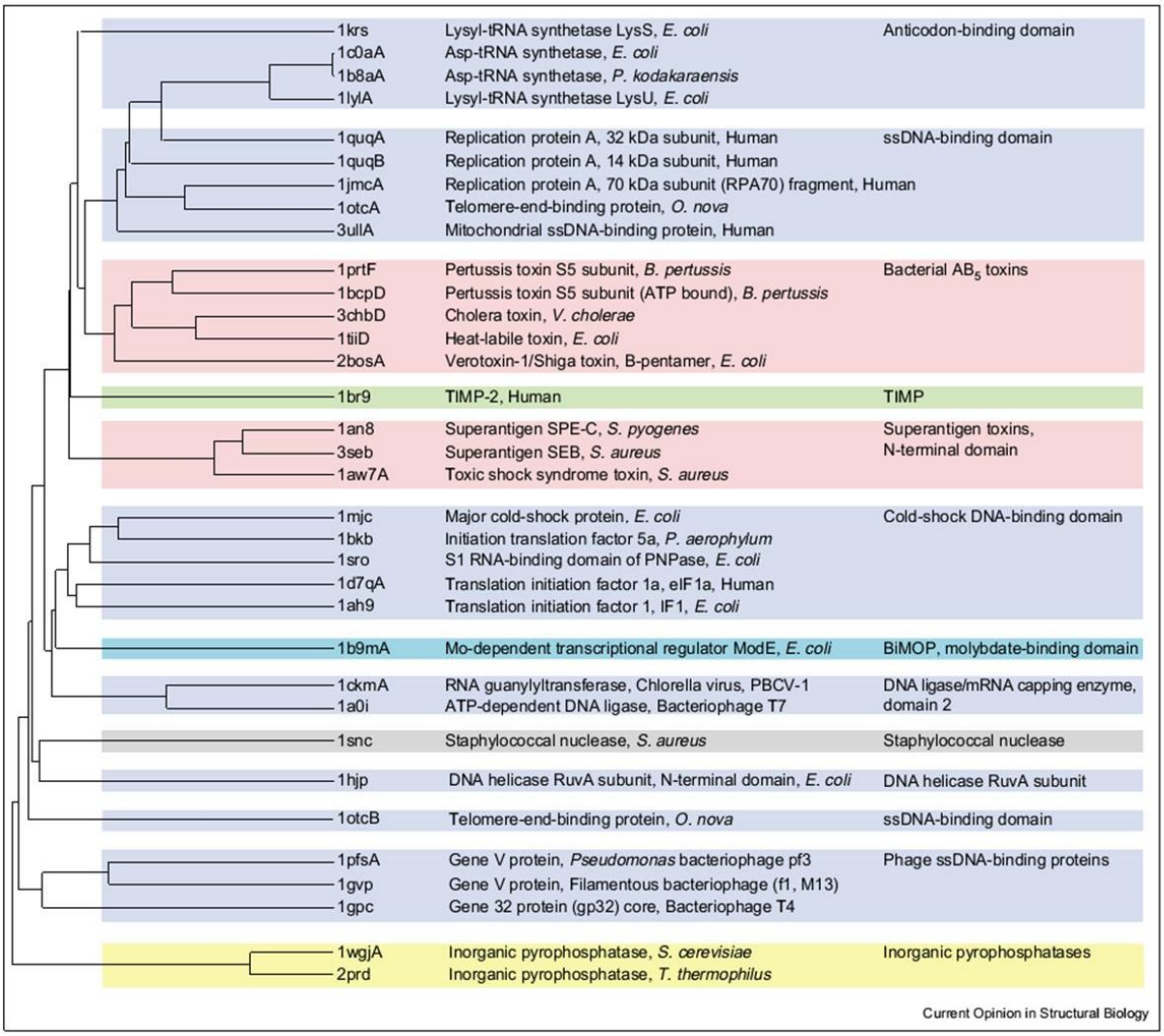

Fig. 1.2. Structural classification of a few OB-fold proteins. A dendrogram of proteins having OB-folds and classified into eight distinct superfamilies is shown. The structural classification of the proteins (SCOP) is given on the right and the PDB reference on the left. SCOP families that belong to the same superfamily are given in the boxes of same colour. (Adapted from Arcus, 2002, Curr. Opn. Str. Biol. 12:794-801)

Another evolutionary linked set of proteins in the nucleic-acid binding superfamily is the novel bacterial OB-fold (BOF) proteins present in several pathogenic bacteria, but with unknown function. Their presence in mobile genetic elements suggests a possible role in bacterial pathogenicity (31).

The oligomerisation of SSB is essential for bringing together the four DNA-binding (OB) folds in an active conformation (32). The C-terminal region of SSBs plays an important role in tetramerization (33). In the typical eubacterial SSB, such as *E. coli* SSB, each monomer has an OB-fold and upon tetramerization of SSB, it results in total availability of four OB-folds (6). However, there are exceptions to this rule, e.g. the SSB proteins of *Deinococcus*–thermus genera form dimers, but each monomer has two OB-folds resulting in a total of four OB-folds (34, 35). The non-eubacterial system possesses SSB with a quaternary structure distinct from that of bacterial SSBs, e.g. heterotrimeric eukaryotic Replication protein A (RPA) (36), and monomeric or dimeric bacteriophage and viral SSB proteins (37). The archaebacteria, *Sulfolobus solfataricus* has a tetrameric SSB similar to eubacterial SSBs, though the DNA binding domains are similar to RPA (38). An overview of the domain structure of ssDNA binding proteins of different bacteria belonging to Eubacteria, Crenarchaea, Euryarchaea and Eukarya genera is shown in **Fig. 1.3**. The number of OB-fold domains varies in different organisms as shown in **Fig. 1.3**, but at least one OB-fold is present in each of the SSB proteins. Some of them also possess a Zn-finger motif **(Fig. 1.3)**.

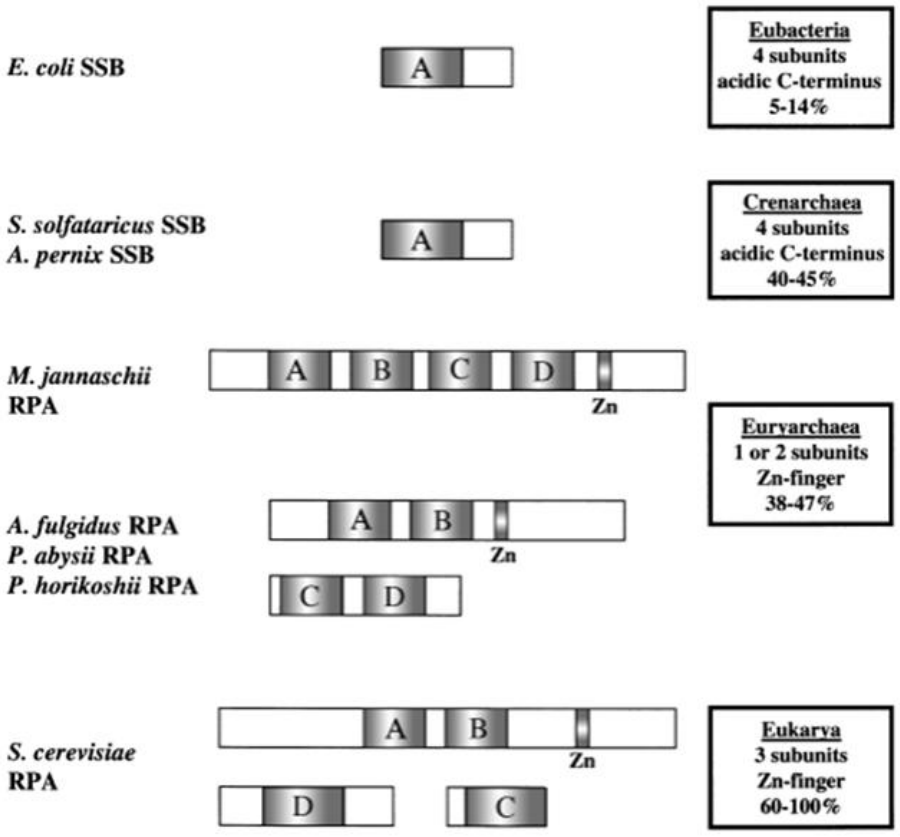

Fig. 1.3. Representation of the domains of different ssDNA binding proteins. DNA binding domains are shown in shaded box and position of the Zn-finger motif is also indicated. (Adapted from Haseltine and Kowalczykowski, 2002, Mol. Microbiol. 43: 1505-1515.)

1.4 INTERACTION OF SINGLE STRANDED DNA BINDING PROTEIN WITH ssDNA

The ssDNA binding site of EcoSSB lies within the first 115 amino acids (33). A series of chemical modification and fluorescence quenching studies indicate a major role for lysine and tryptophan residues of EcoSSB in binding with ssDNA. Of the four tryptophan residues, Trp40 and Trp54 have been found to be involved in stacking interactions with DNA and in high affinity binding to ssDNA (39). In general, the eubacterial SSB proteins bind DNA in a co-operative manner (39) which is not displayed significantly in eukaryotic SSBs (36). The presence of four ssDNA binding sites in the EcoSSB tetramer allows it to bind long stretches of DNA and depending on the number of OB-folds interacting with ssDNA, the binding modes may also differ, and this in turn is dependent on the salt concentrations (40, 41). The major binding modes are $[SSB]_{35}$, $[SSB]_{56}$ and $[SSB]_{65}$, the subscripts indicating the number of nucleotides covered by the SSB complex (39). In $[SSB]_{35}$ binding mode which is more prevalent at low salt concentrations and high protein to ssDNA ratios, it involves binding of only two OB-folds or two subunits to the ssDNA and thereby occluding ~35 nucleotides (42). This results in high unlimited and positive co-operativity, and thus the formation of clusters of protein along the ssDNA resembling a nucleosome (43). This tendency is not shown in $[SSB]_{65}$ binding mode, which is predominant at high salt concentrations and at relatively lower protein concentrations (42). In this mode the SSB tetramer binds to ssDNA using all the four subunits with around 65 nucleotides of ssDNA wrapped around the tetramer (39). A schematic representation depicting the two modes of SSB-binding to ssDNA as predicted earlier (39) is shown in **Fig 1.4**. The different modes of binding may be used

selectively during replication, recombination and repair *in vivo*, though this needs to be proven categorically (6).

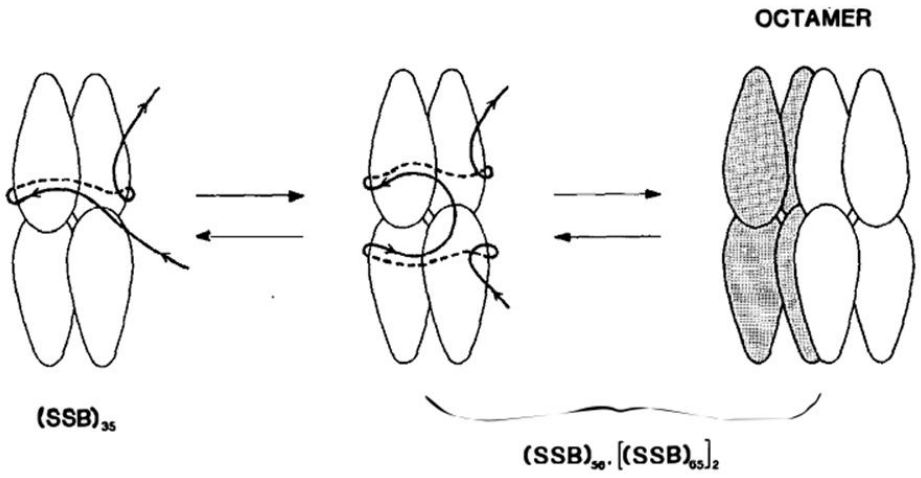

Fig. 1.4 Depiction of SSB_{35} and SSB_{60} binding modes. The interaction of ssDNA with the OB-folds of the SSB tetramer in SSB_{35} and SSB_{60} binding modes is indicated in the form of a cartoon. (Adapted from Lohman and Ferrari, 1994, Annu. Rev. Biochem., 63:57-70)

1.5 INTERACTION OF SSB WITH DNA METABOLISM PROTEINS

The interaction of EcoSSB with several DNA metabolism proteins has been extensively studied and reviewed (6). This interaction requires the C-terminal acidic tail region of SSB (SSB-Ct), suggesting a conserved mechanism by which proteins can recognize and bind to SSB (6, 44). The C-terminus of EcoSSB ends in a highly conserved Asp-Phe-Asp-Asp-Asp-Ile-Pro-Phe sequence. Due to the high density of Asp residues, this region is often referred to as an "acidic tail", and this along with the nature of this hydrophobic region is critical for SSB-protein interactions (45). The conservation of these residues across bacterial species suggest similar role for the C-terminus of other bacterial SSBs as well (6), but not so in eukaryotic SSBs (36). Mutational analysis as well as use of peptide mimics further confirms the role of these residues, especially the C-terminal most 'Phe', in interacting with other proteins (6, 44, 45). In contrast to the conformationally stable and compact OB-fold, C-terminus is very dynamic and susceptible to rapid proteolysis which is stimulated by the binding of DNA.

Several proteins have been identified which directly or indirectly interact with SSB, and these include Exonuclease IX, bacteriophage N4 virion RNA polymerase, DNA Polymerase III, DnaA etc., which are involved in various DNA metabolism pathways and have been discussed in the next section. Though, a defined interaction between each of these proteins and SSB has not been identified. SSB-Ct is expected to mediate these interactions (6). Proteomic approach using either dual affinity tagged proteins or hexahistidine affinity-tagged variants of major *E. coli* proteome also contributed in identifying a few proteins which interact with SSB, and this included peptidase D, and a

putative helicase RhlE, RecG, TopoI, Topo IV etc. (6). Surface Plasmon resonance studies revealed a salt-dependent interaction of the different proteins with SSB-Ct (6).

The most well studied of all protein interactions with SSB is that of RecA, wherein SSB influences RecA activity during DNA repair as well as recombination, and this interaction is dependent on the mode of binding of SSB i.e. $(SSB)_{65}$ or $(SSB)_{35}$ (6). The binding of RecA to ssDNA occurs in two phases, namely nucleation and filament extension, which occurs from 5' to 3' direction. As the filament extension proceed at a high rate, disassembly of RecA from 5' proximal end also occurs, but at a relatively slower rate allowing the assembly end to catch up with the disassembly end (46). This extension is stalled or impeded in the presence of secondary structures. As the RecA filament proceeds, RecA displaces SSB (46). This requires the SSB to be in the totally wrapped up i.e. $(SSB)_{65}$ mode, since the co-operativity is low in this binding mode (40). On the other hand, during strand exchange, wherein the RecA coated ssDNA aligns with the homologous duplex DNA and replaces one strand of the duplex, it requires SSB to be bound to DNA in $(SSB)_{35}$ mode (47). This is because in this mode, two OB-folds per SSB tetramer are unoccupied and these can interact with the ssDNA being generated during strand exchange, facilitating the process (47). Nucleation of RecA on ssDNA, which is a slow process and impeded by the bound SSB, is facilitated by protein mediators, such as RecFOR complex, or individual RecF, RecO and RecR proteins, which can interact with SSB and facilitate its displacement from ssDNA to allow RecA nucleation (48).

1.6 ROLE OF SSB IN DNA REPLICATION

SSB plays a role both in initiation as well as in continuation of DNA replication. In both *E. coli* and *B. subtilis*, the binding of SSB to DnaA enhances the unwinding activity of DnaA at the replication origin, resulting in creation of a larger unwound region for DNA polymerase to start DNA synthesis (49). EcoSSB directly interacts with at least two key proteins during DNA replication namely: the χ-subunit of the replicative DNA polymerase III (DNA Pol III) holoenzyme and primase. Coating of ssDNA with SSB enhances the interaction of the χ-subunit of DNA Pol III with ssDNA as well as SSB (50, 51). The major role of SSB during DNA replication involving DNA Pol III is through the Primase. Upon interaction with SSB, the ability of Primase to latch on to the RNA primer in the lagging strand is enhanced. However, in the presence of DNA Pol III, which also interacts with SSB, the interaction between SSB and primase weakens, thereby decreasing the hold of primase on the primer of the lagging strand. This allows displacement of the primase by DNA Pol III in the lagging strand, thereby ensuring continuation of DNA synthesis (52). Point mutants of χ-subunit, which fail to interact with SSB also impede DNA replication, thus confirming the requirement of their interaction for DNA synthesis both in the leading and lagging strands and thus the maintenance of replisome (53).

Mutational analysis of SSB-Ct reveals that the interaction between χ-subunit and SSB occurs at the C-terminus of SSB. The hydrophobic pocket of χ-subunit of DNA Pol III accommodates the last two amino acids of SSB i.e. P176 and F177 (54). The interaction is similar to that observed between the C-terminus of each of the subunit of SSB tetramer and the 3'→ 5' ssDNA degrading ExonucleaseI (55). Similarly in *B.*

subtilis, the DNA polymerase, DnaE interacts with SSB to promote DNA replication (56). In eukaryotes, the RPA protein also aids in the synthesis during replication by preventing formation of secondary structures as well as interacting with various proteins involved in replication (57).

SSB is also involved in DNA replication restart through its interaction with PriA, a 3'→ 5' DNA helicase, RecG and RecQ DNA helicase whenever the replisome stalls at or dissociates from the replication fork (56, 58). Upon recognition of a stalled DNA replication fork structure, PriA nucleates assembly of the primosomal complex. The activation of the PriA helicase activity upon interaction with SSB allows DNA unwinding; aided further by interaction of SSB with RecG and RecQ and thereafter further strand synthesis with the help of DNA Pol III (53). RPA protein also plays a role in replication restart. The *Methanobacter thermoautotrophicus* RPA interacts with the DNA repair helicase Hel308 *via* its C-terminus, aiding in localization and loading of helicase to the site of activity at blocked replication forks and stimulating its helicase activity (59). The heterotrimeric RPA which co-precipitates with RadA, also helps in resolving the holliday junction intermediates in *Pyrococcus furiosus* possibly by stimulating strand exchange activity or by interaction with a recombination protein Hjc and DNA polymerase and primase (60).

1.7 ROLE OF SSB DURING DNA REPAIR

DNA repair is essential for maintaining the fidelity of genomes and this process requires the presence of single-stranded region in DNA in most cases. SSB which

interacts with ssDNA, also interacts with several of these DNA repair enzymes stimulating their activities as well as localizing them to the site of repair (6).

(a) Recombinational repair: Recombinational repair in both *E. coli* and *B. subtilis* is assisted by SSBs *via* interaction of the DNA repair protein with the SSB-Ct. This enables the localization as well as stimulation of activity of the DNA repair proteins at the site of repair (6). Two key recombination initiation proteins RecQ DNA helicase and RecJ nuclease are involved in the RecF recombination pathway and are known to interact with SSB (61). The helix sub domain of RecQ interacts with the C-terminus of SSB stimulating the RecQ mediated DNA unwinding (61). Binding of SSB also stimulates the RecJ ssDNA binding and the 5' $\rightarrow$ 3' exonuclease activity aiding in its participation in homologous recombination and mismatch repair (62). Interaction between RecJ and SSB aiding in recombinational repair has also been shown for *Haemophilus influenzae* (63).

Upon DNA damage, the resulting regions of ssDNA are immediately covered with SSB to prevent degradation by nucleases. However, this affects the RecA-mediated recombination by impeding RecA nucleation onto the repair site. This is alleviated by specialized proteins called RecA mediators such as RecO, which is part of the RecFOR complex in the RecF pathway (64, 65). In the RecFOR complex, RecR binds both RecF and RecO, while RecO binds ssDNA, RecR and to the C-terminus of SSB (66). The SSB is displaced from ssDNA in the presence of RecO. However, the ssDNA-dependent ATPase activity of RecA, essential for the repair pathway remains inhibited (67). This activity is restored upon binding of RecR, which in turn affects the binding of RecO with ssDNA (67). Thus, SSB mediates the RecF pathway of recombinational repair.

(b) Mismatch repair: One of the important proteins involved in mismatch repair is Exonuclease I (Exo I) which degrades ssDNA in a 3'→ 5' direction in a progressive manner (68). In general, the degradation of ssDNA ends by Exo I would decrease the efficiency of recombination at such DNA structures (69). This necessitates a strong reduction in its activity during DNA recombination (70). However, during mismatch repair the Exo I activity becomes essential (71). The stimulation of the deoxyribophosphodiesterase activity of Exo I at apurinic and apyrimidinic sites (72) and the 3'→ 5' ssDNA exonuclease activity occurs upon binding to the C-terminus of SSB (44, 73). This allows removal of the defective base and facilitates repair.

(c) Base excision repair: SSB also interacts directly with uracil DNA glycosylase (UDG), which catalyzes the first step in base excision repair i.e. removal of uracil from DNA, thus creating an abasic site, which is then degraded and resynthesized (74). This interaction between UDG and SSB is widely conserved across species, but does not work on heterologous bases (75). The interaction localizes UDG to the replication fork potentially and aids in the removal of the misincorporated dUTP (6). UDG is also involved in the generation of ds breaks in case of two closely spaced uracils, which then becomes a target for repair (76). However, this depends on the type of ssDNA substrates and the stimulation of the activity ranges from 7-140-fold (77).

(d) SOS Response: DNA polymerase II (Pol II) is a DNA repair polymerase that is induced early in SOS response (78). Pol II is involved in repair and synthesis of several lesions created specifically during repair of UV-damaged DNA or replication (79, 80). SSB supports binding of Pol II to ssDNA and stimulates its nuclease activity (3, 81). Pol II, by itself exhibits poor processivity, but in complex with SSB, the processivity is

greatly enhanced and it allows bypassing the abasic sites (82, 83). DNA polymerase V (Pol V), which carries out translesion synthesis on damaged DNA (84) requires both SSB and RecA for its activity (85). Binding with the C-terminus of SSB, enhances the access of Pol V to the 3' end of a DNA gap flanked by RecA filaments (86).

SSB C-terminal interactome using GFP fusions and Tap-tags in *B. subtilis* identified several proteins such as DnaE, SbcC, RarA, RecJ, recO, XseA, Ung, YpbB and YrrC in addition to PriA, RecG and RecQ (55), several of which are similar to those already identified and reported from *E. coli* (6).

1.8 ROLE OF SSB IN AIDING TRANSCRIPTION

Single-stranded regions in DNA are also generated during transcription of genes and, hence SSB would be expected to play a role in transcription as well. *In vitro* studies using bacteriophage N4 virion RNA Polymerase and dsDNA promoters indicate specific requirement of EcoSSB (87). EcoSSB bound specifically to 100 bp hairpin regions in the template strand, thereby stabilising small hairpins and generating a secondary structure enabling binding of RNA Polymerase (87). Thus, it is possible that in spite of non-specific nature of binding of SSB to ssDNA, there may exist some specific determinants as well.

Sulfolobus solfataricus SSB interacts with RNA polymerase (RNA Pol) *via* its C-terminal tail, stimulating its activity under TBP (TATA-Binding Protein) limiting conditions (88). The SSB aids in the melting of AT-rich promoter sequences by stabilising the generated ssDNA and allowing RNA Pol access to those genes. SSB may

also aid in the formation of the pre-initiation complex at stalled promoter sites of archaeal chromatin, thereby rescuing transcription from repression (88).

Thus, it can be summarised that SSB proteins play a crucial role in various processes of DNA metabolism i.e. replication, recombination, repair and transcription. This requires both the domains of SSB to be active. The N-terminal domain which is involved in binding to ssDNA is required to prevent formation of secondary structures and to protect ssDNA from nuclease digestion. The C-terminal acidic tail (Ct) which interacts with various proteins is required for stimulating their activity as well as enabling their localisation and assembly at the site of action.

1.9 POSTTRANSLATIONAL MODIFICATION OF BACTERIAL SSB AND EUKARYOTIC RPA PROTEINS

Post-translational modification of SSB proteins has been reported across prokaryotic and eukaryotic systems. The phosphorylation of bacterial SSBs was identified for the first time in *Streptomyces* sp. (89). The *B. subtiilis* BsSSB is phosphorylated by a protein tyrosine kinase YwqD, which could also phosphorylate *E. coli* and *Streptomyces coelicolor* SSB, and is dephosphorylated by the YwqE phosphatase (89). The ability of *B. subtilis* YwpD to phosphorylate the heterologous SSBs suggests the conservation of the phenomenon of the post-translational modification of SSBs of bacterial species (89). The phosphorylation occurred at a well conserved Tyr residue occupying similar positions and structural motif in most bacterial species (90). The phosphorylation of BsSSB enhances binding to ssDNA by about 200-fold (89). However,

the level of the phosphorylated SSB decreases upon DNA damage, suggesting a possible role of phosphorylation-dephosphorylation in regulating DNA repair (89).

The post-translational modification has been more than extensively studied in the eukaryotic counterpart, RPA. It is subjected to multiple post-translational modifications, which includes phosphorylation at Ser and Thr residues, poly-ADP ribosylation and SUMOylation, thereby regulating its activity in terms of binding ssDNA as well as in interacting with other proteins (91). The hyperphosphorylation of RPA is induced in response to DNA damage and of the three subunits of RPA, p34 and p70 have phosphorylation sites, while p14 has none (92). The hyperphosphorylation of p34 causes a conformational change in the DNA binding domain located in RPA70 (p70) (92). The cell-cycle dependent phosphorylation occurs specifically at p34. During mitosis, RPA32 is inactivated by hyperphosphorylation, resulting in RPA disassembly from chromatin (93). In addition RPA is hyperphosphorylated in response to DNA damage by the kinases, ATM (Ataxia Telangiectasia Mutated**)**, ATR (ATM and Rad3-related) and DNA-Protein Kinase. The 70 kDa subunit of RPA associates with SUMO-specific protease and maintains a hyposumoylated state during S-phase (94). The SUMOylation of RPA70 has been found to be essential for recruitment of Rad51 at the foci of damage to initiate DNA repair and thus plays a crucial role in regulating DNA repair through homologous recombination (94).

1.10 EFFECT OF MODULATION OF SSB LEVELS ON *E. coli* PHENOTYPE

Several viable and non-viable mutants of *ssb* gene of *E. coli* were generated and reviewed extensively (95). The major *ssb* mutants with the observed phenotype as cited in the review article (11) are listed in **Table 1.1.**

Table 1.1: List of *ssb* mutants of *E. coli* and their phenotype

Mutation	**Site of mutation**	**Phenotype**
ssb1	55 His → Tyr	Defect in DNA Replication
ssb2	55 His → Tyr	Temperature sensitive
ssb3	15 Gly → Asp	UV sensitive, No effect on replication
ssb113	176 Pro → Ser	Temperature, UV and salt sensitive
ssb114	176 Pro → Ser	UV and MMS sensitive
*ssb*W40F	40 Tyr → Phe	Temperature sensitive
*ssb*W54F	54 Tyr → Phe	Temperature sensitive
*ssb*W80F	80 Tyr → Phe	Temperature sensitive
*ssb*F60A	60 Phe → Ala	Temperature sensitive
*ssb*H55L	55 His → Leu	Temperature sensitive
Δ*ssb*	Deletion of the gene	Lethal. Survived only if SSB expressed in *trans*
*ssb*P176S	176 Pro → Ser	Temperature sensitive, Decreased binding to other proteins through Ct

Most *ssb* mutants are found to be temperature sensitive for DNA replication (95) and defective in several DNA repair processes, UV sensitive, defective in RecA induction, Weigle reactivation and mutagenesis, exhibit excessive post-irradiation DNA degradation and deficiency in recombination *in vivo* (11). The P176S mutation in *ssb* causes decrease in interaction of SSB-Ct with other proteins (96). It also exhibits temperature sensitivity at 30°C which could not be suppressed by overexpression of the mutant protein, unlike most other *ssb* mutants (96). The mutant exhibits defects in DNA synthesis and increase in double strand breaks which eventually leads to chromosome degradation (97). The block in induction of SOS at 30°C results in increased UV-sensitivity, but this could be suppressed by introduction of a *recA* allele (*recA*-E38K), suggesting a direct role for SSB in RecA activation for SOS induction and SOS mutagenesis (98). The *ssb*1 mutation refers to a point mutation in the OB-fold (H55Y), a temperature-sensitive phenotype and complete loss of activity at 42°C which could be suppressed by overexpression of the mutated protein (99). The mutant also destabilized the SSB tetramer, which in turn could be responsible for the observed defects in DNA repair (99).

While deletion or mutation of *ssb* gene cause lethal or temperature sensitive phenotype (6, 11), overexpression of the protein also affects the physiology of *E. coli*. The most prominent change observed upon overexpression of SSB is the decrease in levels of LexA in *E. coli* resulting in a differential increase in expression of SOS regulon genes (100). It also affects DNA repair in a differential manner, enhancing the efficiency of excision repair, while decreasing that of recombinational repair (100). This is achieved through modulating RecA activity *in vivo*, allowing formation of short complexes of

RecA and ssDNA, thereby facilitating excision repair, but delaying the formation of long nucleoprotein complexes, thereby slowing down recombinational repair (100). SSB overexpression also affects DNA repair post exposure to UV-radiation. Due to the delayed formation of long nucleoprotein complexes, the second phase of repair of UV-damaged DNA is affected. This slows down the overall repair of UV-damaged DNA, thereby decreasing cell viability (101). The phenotype of SSB overexpression being similar to that of *recF* mutation in *E. coli*, suggested the possibility of RecF overexpression aiding RecA in displacing SSB from ssDNA (101). The interaction between RecA, RecF and SSB is further substantiated by the observation that the *recF* mutation in *E. coli* can be suppressed by a specific *recA* mutation (*recA*441) so as to alleviate UV-sensitivity, but could not do so if SSB was overexpressed (102). SSB overexpression enhances the frequency of UV-induced mutation in $recA^+$ background, due to decreased photoreactivation (103). In a $recA^-$ background, SSB binds thymidine dimers initiating photoreactivation and thereby reducing the frequency of mutation (104).

1.11 ORGANISMS WITH MULTIPLE SSB PROTEINS

The SSB proteins have a multitude of roles in the cell due to their ability to non-specifically bind ssDNA. In some organisms, due to the requirement of specialized SSBs, alternative SSBs have also evolved resulting in multiple genes coding for SSB or SSB-like proteins. Presence of multiple SSB paralogs is not very rare in bacteria and has been studied in *B. subtilis* and *Deinococcus*. In *B. subtilis*, one SSB protein (SsbA) is similar to *E. coli* SSB having an N-terminal OB-fold and a C-terminal tail for interaction with other proteins (105), while the second SSB (SsbB), coded by *ywpH* gene, is 113 amino acids

long and contains an OB-fold, but lacks the C-terminus found in the primary SSB (106). Both the proteins are capable of binding ssDNA. SsbA is expressed maximally in logarithmic phase in actively replicating cells, while SsbB is expressed mainly in stationary phase (107). SsbB performs a specialized function of protecting internalized ssDNA during natural transformation (106, 107). Such SSBs have been found in several other naturally transformable bacteria as well (105). The *ssbA* gene mutants are lethal, while *ssbB* gene mutants are viable, indicating that SsbB performs a function distinct from SsbA and cannot complement *ssbA* mutation, which performs the role of primary SSB. The *ssbB* gene mutant, are however, 50-fold less competent than the wild type cells (106).

D. radiodurans also contains at least two SSBs. The primary SSB contains two OB-folds per monomer involved in binding to ssDNA and dimerisation, and a C-terminal tail that directs the interactions with other proteins (108). The second SSB is designated as DdrB which is not expressed under normal conditions, but is induced in response to ionising radiation (109). DdrB shows domain similarity with other bacterial SSBs, but differs in forming a stable pentamer arranged in a ring structure (109). The crystal structure of DdrB confirmed its existence as a pentamer, and this novel fold possibly aids in binding to damaged DNA and subsequent recovery (110). An extended DNA binding surface along the top surface of DdrB pentamer (110) is involved in an early step of DNA double strand break repair i.e. extended synthesis dependent strand annealing (ESDSA) and plasmid transformation because of its single-strand annealing activity (111). DdrB is unable to complement mutation in *ssb* of *D. radiodurans* and the radiation sensitivity of *ddrB* mutant is not complemented by overexpression of SSB indicating, specific and

independent roles for the two SSBs (112). Decrease in the inherent levels of SSB decreased the radiation resistance of *D. radiodurans*, suggesting requirement of a minimum level of SSB for radiation protection (112).

All eukaryotic cells have at least two compartmentalized SSBs: RPA found in the nucleus and mtSSB found in mitochondria (113, 114). They perform analogous functions in their respective compartments, are not evolutionary related, and mtSSB are homologues of eubacterial SSBs (115). The mtSSB has an extended N-terminal domain, followed by an OB-fold domain but lacks the amphipathic C-terminus, but interacts and modulates the activity of two proteins, namely DNA polymerase γ and mtDNA helicase. Deletion of mtSSB from the mitochondria results in a loss of mitochondrial DNA replication and eventually mitochondria loss (115).

The *ssb* gene organization of 87 bacterial genomes was compared with that of *E. coli* and *B. subtilis* (105). All genomes showed presence of at least one *ssb* homologue with 15 genome sequences showing *ssb* gene order similar to *E. coli* (i.e. *ssb* gene divergently situated to the *uvrA* gene), while 35 genome sequences showed an gene alignment similar to *B. subtilis* (*ssb* gene flanked by *rpsF* and *rpsR* genes). Classification of different *ssb* genes across bacterial species based on their organization in listed in **Table 1.2**.

1.12 REGULATION OF SSB EXPRESSION

The *ssb* gene of *E. coli* is preceded by three promoters. Of these, the upstream most promoter is inducible by DNA damage and has a LexA binding site (116). The *uvrA* gene, also regulated by LexA is adjacent to *ssb*, but transcribed in the opposite direction,

and thus a common LexA binding site controls the divergent expression of *ssb* and *uvrA* genes (117). Upon SOS induction, the level of SSB did not increase significantly over a 1 h period, but a slow increase in the relative rate of synthesis of SSB was observed. A two-fold increase in SSB level was observed after 3 h exposure of mitomycin C, but not earlier (118). Thus, *ssb* induction is considered to be very slow in the RecA-LexA mediated SOS regulon.

Table 1.2 Classification of *ssb* genes based on gene organization

Class	Gene Organization	Multiple *ssb*s	Gram stain	Number of Species	Examples
I	*rpsF-ssb-rpsR*	+	+/-	22	*Bacillus subtilis*
II	*rpsF-ssb-rpsR*	-	+/-	13	*H. pylori, Borrelia burgdorferi*
III	*uvrA-ssb*	-	-	15	*E. coli, Salmonella typhimurium*
IV	Variant	+	-	19	*Xanthomonas citri, Anabaena* 7120, *Xylella fastidosa.*

In *D. radiodurans*, two 17 bp conserved radiation/desiccation response (RDRM) motifs i.e. 5'-TTATGTCATTGACATAA-3' (RDRM1) and 5'-AACCGCCATCGCCAGCA-3' (RDRM2) upstream of *ssb* gene were found to act as regulatory palindrome and inhibit the *ssb* promoter activity (119). Exposure to γ-

irradiation or mitomycin C stresses activated the transcription of the *ssb* gene (119). Similar palindromes are also found to act as *cis*-elements up-regulating transcription of certain eukaryotic genes. A third form of regulation of SSB is exhibited at translational level in *E. coli*, wherein SSB negatively regulates and inhibits its own translation by binding to the *ssb* mRNA (120).

1.13 CYANOBACTERIA

Cyanobacteria (blue-green algae) are phylogenetically a primitive group of Gram-negative prokaryotes which appeared on earth over 3 billion years ago (121). They were probably the primary producers of organic matter and the first organisms to release oxygen into the oxygen-free atmosphere by oxygenic photosynthesis like higher plants (122). Thus, cyanobacteria were possibly responsible for a major global evolutionary transformation leading to the development of aerobic metabolism and the subsequent rise of higher plant and animal forms (123).

Cyanobacteria were also known as blue-green algae due to presence of blue pigment phycocyanin and green pigment chlorophyll *a* (124). They were classified as cyanobacteria due to prokaryote like cellular architecture (125). However, they also resemble plants because of structural similarity of membrane and oxygen evolving apparatus, lipid composition, and assembly of proteins (126) as well as similar photochemical reactions (127). Chloroplasts found in plant cells are believed to have evolved by endosymbiosis of cyanobacteria-like ancestors by eukaryotic cells (128), hence they may act as the connecting link between bacteria and plants. All cyanobacteria

are photoautotrophic organisms, yet many can grow heterotrophically, using light only for energy and organic compounds as a carbon source (129).

Cyanobacteria are classified on the basis of their morphology as: unicellular (e.g. *Synechococcus* sp.) or filamentous (eg. *Anabaena* sp.), non-heterocystous (e.g. Leptolyngbya nodulosa) or heterocystous (e.g. *Anabaena* sp.), both capable of fixing atmospheric nitrogen, and unbranched (*Anabaena* sp.) or branched (e.g. *Tolypthorix, Stigonematales*) filaments (130). Cyanobacteria occupy a broad range of ecological niches which include hot springs (*Synechococcus* sp., ~70°C;, *Oscillatoria terebriformis*, ~54°C, Antarctic and Arctic regions (*Calothrix parentina, Phormidium frigidum*); oceans (*Synechococcus elongatus*), fresh water lakes (*Nostoc* and *Anabaena* spp., *Microcystis aerugenosa*), deserts (*Gloeocapsa* sp.); and as symbiotic relationship with fungi and plants (*Anabaena azollae*) suggesting high variability in adapting to diverse environmental factors (131, 132).

Cyanobacteria are characterized by their general ability to perform oxygenic photosynthesis and fix CO_2, but some of them also carry out oxygen sensitive nitrogen-fixation. The enzyme Nitrogenase, which converts atmospheric nitrogen (N_2) into Ammonia (NH_3), is sensitive to oxygen. This problem is overcome by adapting to either temporal or spatial separation (133). Temporal separation refers to alternating photosynthesis and nitrogen-fixation depending on light-dark cycles (134, 135). In spatial separation, some filamentous strains develop the ability to terminally differentiate vegetative cells (which carry out photosynthesis) into heterocysts, which are less pigmented cells, large, thick walled to maintain anaerobic interior by reducing gas diffusion (136, 137). At the junction of vegetative cells and heterocyst, polar bodies are

formed due to thickening of cell wall and deposition of pigment cyanophycin, which acts as nitrogen reserve. In contrast to vegetative cells, the environment inside heterocyst is microaerophilic or anaerobic, photosystem II is inactivated and traces of Oxygen is removed primarily by respiration (136). Heterocysts receive their nutrients, electron transporters mainly in the form of sugars from vegetative cells and in return supply nitrogen in form of glutamate through glutamate synthetase or glutamate synthase pathway (ammonia $\rightarrow$ glutamate) to vegetative cells (136, 138). In anaerobic cyanobacteria (*Plectonema*, *Synechococcus*), nitrogenase is induced only under anoxia or low oxygen condition or sulphide which inhibits oxygenic photosynthesis.

As a group, cyanobacteria are thought to have survived a wide spectrum of environmental stresses, such as heat, drought, salinity, photo oxidation, anaerobiosis, and osmotic and ultraviolet and ionizing radiation stress (122, 139). Adaptation to unavoidable environmental stress is crucial for the survival of all living organisms. Under extreme abiotic stress conditions, organic molecules such as lipids, proteins, and nucleic acids are prone to damage and/or degradation (140). Cyanobacteria, like other organisms, respond to exposure to abiotic stress by inducing a set of proteins (141), which help acclimatize the cells to these stresses (142).

1.14 CYANOBACTERIAL RESPONSE TO DNA-DAMAGE INDUCING STRESSES

(a) Response to mitomycin C

Mitomycin C is a potent DNA cross-linker known to be capable of inducing the SOS response system in many bacterial species (143) and has been used to induce

cyanobacterial prophages in environmental samples (144). Genes encoding RecA, LexA, UmuC and UmuD are significantly unregulated under mitomycin C, while several SOS response genes are found to be transcriptional activated after mitomycin C treatment in marine *Synechococcus* sp. (145). A rapid increase in transcript as well as protein levels of RecA is observed after mitomycin C treatment in the nitrogen-fixing *Anabaena variabilis* (146).

(b) Response to UV radiation

Being a primitive photosynthetic oxygen-evolving prokaryotes, cyanobacteria encountered extreme ultraviolet (UV) radiation possibly due to the absence of ozone layer billions of years ago. UV radiation (UVR) is composed of UV-C (200-280 nm), UV-B (280-315 nm) and UV-A (345-400 nm) in the natural environment. Of this UV-B has higher deleterious effect on living organisms due to its absorption by biomolecules resulting in direct damage or indirectly through reactive oxygen species (ROS) causing lethal effects on biological systems (147). UV-A radiation, which is not absorbed directly by the DNA, can still induce DNA damage either by producing a secondary photoreaction of the existing DNA photoproducts or *via* indirect photosensitizing reactions (148).

Since, both photosynthesis and nitrogen-fixation are energy dependent processes requiring solar energy in nature, harvesting of this solar energy results in exposure of cyanobacteria to UV-A and UV-B in their natural habitat (149). UVR affects the growth, photosynthetic activity, trichome morphology and heterocyst differentiation in *Anabaena* 7120 (150). It also affects pigmentation (151), nitrogen metabolism (152), nitrogenase

activity and CO_2 uptake (153). Exposure to UV-B radiation also affected the protein profile of *Anabaena* possibly due to the inhibition of translational activity as a result of the oxidation of EF-G (154), as also observed in *Synechocystis* PCC6803 (155). Cyanobacteria have developed several lines of defense mechanism to cope with UVR. This includes (i) migration from high to low UVR levels in the water column, (ii) formation of mats, (iii) different changes in morphology to increase self-shading (iv) synthesis of extracellular polysaccharides, (v) antioxidant system which includes non-enzymatic [ascorbate (vitamin C), a-tocopherol (vitamin E), carotenoids and reduced glutathione] and enzymatic antioxidants [superoxide dismutase (SOD), catalase, glutathione peroxidase (GSH-Px) and the enzymes involved in the ascorbate–glutathione cycle to detoxify ROS etc., and (vi) repair and resynthesis of DNA (149).

(c) Response to ionizing radiation

The ancient organisms, such as cyanobacteria, have also been exposed to comparatively higher levels of ionizing radiation during their evolution and so have adapted well by developing several defense mechanisms. The unicellular non-nitrogen fixing *Anacystis nidulans* is radiosensitive and exhibits a D_{10} dose of about 0.2–0.4 kGy for gamma rays (156, 157) possibly due to photosynthetic decline due to a defective thylakoid membrane biogenesis (158). On the other hand, cyanobacteria such as *Chroococcidiopsis* and *Nostoc muscorum* have been shown to tolerate >10 kGy dose of ionizing radiations under adiazotrophic growth conditions (159, 160). The nitrogen-fixing *Anabaena* strains exhibit high radioresistance, LD_{50} ~ 6 kGy (161, 162). Although the radioresistant mechanism is not understood completely, but some of the factors could be

(i) redundancy of the genome (163), (ii) efficient DNA repair mechanism resulting in regulated processing of damaged genome (160, 162), and (iii) protein recycling (162).

(d) Response to desiccation

In general the most desiccation tolerant non-spore forming bacteria are usually Gram positive, which also include forms that accumulate Mn(II) resulting in a high Mn(II) to Fe ratio and are also radiation resistant (164). Though, cyanobacteria possess cell wall typical of Gram negative bacteria, they also exhibit phylogenetic similarity with Gram positive bacteria (165). They exhibit high tolerance to both desiccation and gamma radiation stresses (160, 162, 165). Cyanobacteria circumvent desiccation by the formation of spores or akinetes, wherein the cells show an extremely slow growth rate, but remain viable and sporulate upon return to favourable environment or rewetting (166). The extracellular polysaccharide (EPS) also contributes to the desiccation tolerance of cyanobacteria (167). EPS regulates efflux and influx of water from cells and thus protect from cell wall damage against cell swelling and bursting (160). Redundancy of genome has also been considered to cope against DNA damage (163).

1.15 *Anabaena* SP. STRAIN PCC7120

The total genome size of *Anabaena* PCC7120 is 6.41 Mb which includes one chromosome and 6 plasmids, *viz* α (408 kbp), β (186 kbp), γ (101 kbp), δ (55 kbp), ε (40 kbp) and ζ (5.5 kbp). The chromosome has 5368 potential protein encoding genes, four sets of r-RNA genes, 48 t-RNA genes representing 42 t-RNA species and 4 genes for structural RNAs'. Of the 5368 protein coding genes, 1453 were annotated as

'hypothetical genes' and 1519 as 'unknown'. The average gene density is found to be in every 1195 bp. In addition to the most common start codon of ATG, others such as GTG, TTG or ATT codon have also been assigned as start codons in *Anabaena* genome (168). The ORFs are denoted by a serial number with three letters, first representing the species name, **'a'** in case of *Anabaena*, the second letter represents length of the ORF, ORF longer than or smaller than 100 codons designated as **l or s** respectively and the third letter for the orientation of the reading frame i.e. left or right **(l or r)**.

Genetic manipulation techniques are well established for *Anabaena* 7120 which can be achieved by conjugation (169). Mutants can be generated either by homologous double recombination (170) or by conditional lethal approach (171). Thus, *Anabaena* 7120 acts as a suitable model for genetic studies. Genetic tools for plasmid-based recombination has also been developed (172) and attempts have been made to generate recombinant strains of this species tolerant to variety of abiotic stresses such as heat and salinity a stresses (173, 174) making use of an integrative vector pFPN where in a desired gene under the transcriptional control of a strong light-inducible *psbA1* promoter can be introduced at an innocuous site in the genome of *Anabaena* 7120 (172). Also overexpression of proteins in *Anabaena* 7120 using stable plasmids has been reported wherein expression is under the *psbA1* promoter along with co-transcription of *gfp* allowing microscopic visualization of extent of segregation in recombinant *Anabaena* strains (175).

1.16 SCOPE OF THE THESIS

The high radioresistance of *Anabaena* 7120 has been attributed to a robust DNA repair mechanism (162), which is contributed by the ability of the organism to repair single-stranded (ss) and double-stranded (ds) breaks in DNA. SSB being a key protein involved in all DNA metabolic pathways was chosen for present study in *Anabaena* 7120. In the cyanobacterial genome database (http://genome.microbedb.jp/cyanobase/Anabaena), two proteins encoded by genes, *alr0088* (*ssb1*) and *alr7579* (*ssb2*) were annotated as single stranded DNA binding proteins of *Anabaena* 7120 and protein product of another gene *all4779* (*ssb3*) annotated as a hypothetical protein has a ss-DNA binding domain. Presence of multiple *ssb*s would indicate either redundancy or specialised function for each of the protein. Of the three SSB-like proteins, SSB1 and SSB2 were naturally truncated at the C-terminus and possessed only the N-terminal OB-fold, and lacked the C-terminal acidic tail and the intervening P/G-rich region, typical of bacterial SSBs. On the other hand, SSB3, though not annotated as SSB, possessed all the three regions. The present study envisages the following investigations:

(I) Biochemical characterization of the three SSB proteins

(II) Expression and regulation of the three *ssb* genes

(III) Investigation into the possible physiological role for each of the SSB proteins in *Anabaena* 7120.

The thesis is composed of six chapters including the general introduction. The chapters describe the following aspects

Chapter 2 gives complete details about the organisms used and their growth, all the experimental procedures followed, along with a list of various plasmids and strains used and primers designed for the purpose of cloning. It also gives details about composition of various buffers used for Nucleic acid isolation, agarose gel electrophoresis, protein purification, SDS-PAGE electrophoresis and biochemical assays.

Chapter 3 describes the biochemical characterisation of the three SSB proteins of *Anabaena* 7120. *In silico* analysis of the multiple *ssb*-like genes of *Anabaena* 7120 revealed that SSB1 and SSB2 coded by *alr0088* and *alr7579* respectively are 13 and 14 kDa proteins respectively and truncated beyond the N-terminal OB-fold, while SSB3 coded by *all4779* is a 18 kDa protein and possess the OB-fold, P/G-rich linker and the C-terminal acidic tail. Of the three proteins, SSB1 and SSB2 showed better similarity with other bacterial SSBs at amino acid level, compared to SSB3. The individual *ssb* genes were cloned, protein overexpressed and purified from *E. coli* for biochemical analysis. Oligomeric status of the proteins was determined by gel filtration chromatography and glutaraldehyde-based cross-linking. Interaction of the SSB proteins with ssDNA was studied by Electrophoretic mobility shift assay (EMSA) and fluorescence quenching techniques.

Chapter 4 deals with expression studies of the three *ssb* genes in *Anabaena* 7120 exposed to different abiotic stresses. Reverse Transcriptase analysis was carried out to determine whether *ssb3* was a monocistronic gene or part of a multicistronic operon. Of the other two *ssb* genes, *ssb1* is monocistronic based on the gene orientation with respect to its adjacent genes, while *ssb2* is the first gene in a series of genes transcribed in the same direction. The promoter regions corresponding to the three *ssb* genes were

identified and cloned in a promoterless vector, pAM1956. The activity of the promoters in response to mitomycin C, γ-irradiation and desiccation stresses were analysed fluorimetrically. Expression of the SSB proteins in response to the above stresses as well as UV-B stress under nitrogen-fixing conditions was analysed by Western blotting and immunodetection using the corresponding antibodies. EMSA studies were carried out to study the interaction the three promoters with LexA, a known regulator of bacterial *ssb* genes.

Chapter 5 chapter deals with the generation of recombinant *Anabaena* strains with either mutation of one of the *ssb* genes or overexpressing one of the SSB proteins using pAM1956 vector. The survival and tolerance of the recombinant *Anabaena* strains to mitomycin C, UV-B, γ-irradiation and desiccation stresses was analysed by measurement of chlorophyll *a* and colony forming units.

The complete results of the thesis have been summarized in **Chapter 6**, followed by a list of references cited in the text.

CHAPTER 2

Materials and Methods

2.1 BIOINFORMATIC ANALYSIS (SOFTWARES USED)

The different software used for Bioinformatic analysis were as follows: (i) Conserved domain database (CDD) analysis using CDD-BLAST (http://www.ncbi.nlm.nih.gov/Structure/cdd) (ii) extinction coefficient determination using Expasy software (http://web.Expasy.org/protparam) (iii) Illustration of Dendrogram for SSB proteins from various bacterial species using Clustal omega software (http://www.ebi.ac.uk/Tools/msa/clustalo) (iv) putative promoter prediction using online analysis tool (http://molbiol-tools.ca/Promoters.htm).

2.2 BACTERIAL STRAINS AND GROWTH MEASUREMENT

2.2.1 Growth medium and conditions

E. coli cells were grown at 37°C in Luria-Bertani (LB) medium containing appropriate antibiotics **(Table 2.1)** in orbital shaker with shaking at 150 rpm or on LB solid agar (1.2%) plates unless specified otherwise.

Anabaena sp. were grown photoautotrophically in combined nitrogen-free BG-11 liquid medium, pH 7.2 (176) at 27°C under continuous illumination (30 μE m^{-2} s^{-1}) and with either constant shaking at 100 rpm or with aeration at 3 L min^{-1}.

2.2.2 Abiotic stresses applied

Wild type and recombinant *Anabaena* 7120 strains were exposed to different abiotic stress conditions as follows: (i) mitomycin C stress with different concentrations of the mutagen (stock 1 mg mL^{-1} in water) for up to 30 min, (ii) UV-B (280 nm) stress was applied by exposing the cells spread on a nitrocellulose membrane to UV radiation

(dose rate 5 J m^{-2} sec^{-1}) for different time periods, (iii) radiation stress of 6 kGy ^{60}Co γ-rays (dose rate 4.5 kGy h^{-1}), and (iv) desiccation stress was applied by drying the cells on a nitrocellulose membrane by applying vacuum for 1 minute after complete filtration. The filters were then placed either in desiccator having fused $CaCl_2$ or on agar plates in humid chambers, both maintained under sterile conditions.

Table 2.1 Antibiotics and other additives used in growth media

Antibiotics/additives	**Stock Concentration**	**Medium of preparation**	**Final Concentration**
Carbenicillin	100 mg mL^{-1}	St. Distilled water	100 μg mL^{-1}
Kanamycin	50 mg mL^{-1}	St. Distilled water	50 μg mL^{-1}
Chloramphenicol	34 mg mL^{-1}	100% distilled ethanol	34 μg mL^{-1}
Neomycin	50 mg mL^{-1}	St. Distilled water	12.5 or 25 μg mL^{-1}
IPTG	1 M	St. Distilled water	1 mM

2.2.3 Measurement of growth and assessment of tolerance and recovery after abiotic stresses

Growth of *Anabaena* 7120 was measured in terms of chlorophyll *a* content. Chlorophyll *a* was extracted from cyanobacterial culture with 95% methanol and estimated spectrophotometrically ($OD_{665.4}$) (UV-visible spectrophotometer UNICAM,

UK) as described earlier (177). Abiotic stress tolerance was estimated as chlorophyll *a* content and survival in terms of colony forming units (CFUs). For CFU determination, 100 µL culture aliquots were removed at specific intervals during stress and plated on to solid BG-11 agar medium with or without Neomycin ($Neo_{12.5}$), followed by incubation at room temperature under continuous illumination. The colonies were counted after 10 days of incubation and CFUs estimated per mL of culture.

2.3 ISOLATION OF NUCLEIC ACIDS AND THEIR ELECTROPHORETIC SEPARATION

Table 2.2 List of reagents used for nucleic acid purification

Reagent	**Composition**
TES	10 mM Tris-HCl, 25 mM EDTA, pH 8.0, 150 mM NaCl
$T_{10}E_{25}$	10 mM Tris-HCl, 25 mM EDTA, pH 8.0
$T_{10}E_{1}$	10 mM Tris-HCl, 1 mM EDTA, pH 8.0
TE_{100}	100 mM Tris-HCl, 100 mM EDTA, pH 8.0
10X DNA Loading Dye	0.4% bromophenol blue, 0.4% Xylene cyanol, 50% Glycerol
1 X TBE	8.9 mM Trizma base, 8.9 mM Boric Acid, 0.2 mM EDTA
2X RNA Loading Dye (10 ml)	64% Formamide (6.4 ml), 4.4% Formaldehyde (1.2 ml of 37% formaldehyde), 2X MOPS (2 ml of 10X), 8% Sucrose (0.4 ml of 50% sucrose solution), 0.2% Bromophenol Blue, 0.2% Xylene Cyanol

2.3.1 Isolation of plasmid and chromosomal DNA

Isolation of plasmid DNA

Plasmid DNA was isolated using Qiagen Spin Mini Prep Kit, Qiagen (Germany) as per the manufacturer's protocol involving alkaline lysis method. The plasmid DNA was stored in $T_{10}E_1$ buffer **(Table 2.2)**.

Isolation of chromosomal DNA

Ten mL of cyanobacterial culture (10 μg chlorophyll *a* density mL^{-1}) was centrifuged at 6000 rpm for 10 min at room temperature. The pellet was washed once with 10 mL of 1 M NaCl and twice with 20 mL of TES. Each time the cell suspension was centrifuged at 6000 rpm for 10 min and the pellet was finally resuspended in 10 mL of $T_{10}E_{25}$ **(Table 2.2)**. Cells were lysed by adding 1 mL of lysozyme (2 mg/mL in $T_{10}E_1$) **(Table 2.2)** and incubating with gentle shaking at 37°C for at least 1 h, such that the filaments were broken to 2-3 cell stage as observed microscopically. To this freshly prepared 1.2 mL of sarcosyl/Proteinase K mix (130 mg lauryl sarcosine in 0.5 mL of 20 mg mL^{-1} Proteinase K solution, volume made up to 1.2 mL with water) was added and incubated at 37°C for 45 min to 1h. Equal amount of phenol (equilibrated with 100 mM Tris-HCl, pH 8.0) was added to the solution, mixed gently and centrifuged at 5000 rpm to separate the layers. The aqueous layer was further purified by extraction once more with phenol and then with chloroform: isoamyl alcohol (24:1). Chromosomal DNA was spooled after gently mixing the aqueous layer with $1/10^{th}$ volume of 3 M sodium acetate, pH 5.2 and 2 volumes of chilled absolute distilled ethanol. The spooled DNA was then washed with 70% ethanol, air dried, dissolved in $T_{10}E_1$ and stored in 100 μL aliquots at -20°C.

2.3.2 Restriction enzyme digestion and electrophoretic separation of DNA

Plasmid DNA was digested with the specified restriction endonucleases (2 U μg DNA^{-1}) using the corresponding buffer at 37°C for 3 h followed by electrophoresis on agarose gel. The DNA fragment was excised out from the agarose gels and eluted using Qiagen Gel Extraction kit, Qiagen, Germany as per the manufacturer's protocol.

DNA was mixed with appropriate amount of 10X DNA loading dye **(Table 2.2)** and electrophoretically resolved on 0.8-1.0% agarose gels (Sisco Research Laboratories, India) containing 0.05 μg mL^{-1} ethidium bromide prepared in 1X Tris-Borate-EDTA (TBE) buffer. Electrophoresis was carried out at 8 V cm^{-1} and the DNA fragments were visualised using a UV transilluminator. The 1 kb or 100 bp ladder (from Bangalore Genie, India) was used as a DNA marker to calculate the molecular size of the various DNA fragments.

The restriction digested insert DNA was ligated to vector DNA digested with the same set of restriction endonucleases by taking 10-fold molar excess of the insert DNA. The mix along with ligase buffer and 1 U of T4 DNA ligase (Rapid DNA ligation Kit, MBI Fermentas, USA) was incubated at 25°C for 1 h.

2.3.3 Isolation and electrophoretic separation of RNA

Total RNA was extracted from logarithmic phase cultures of *Anabaena* 7120 (10 μg chlorophyll *a* density mL^{-1}) using MN RNA isolation Kit as per manufacturer's manual. The RNA was quantified spectrophotometrically at 260 nm.

RNA sample was mixed with appropriate amount of 2X RNA loading dye **(Table 2.2)** and electrophoretically resolved on 1.2% agarose, containing 0.05 μg mL^{-1} ethidium

bromide prepared in 1X Tris-Borate-EDTA (TBE) buffer. Electrophoresis was carried out at 8 V cm^{-1} and the RNA fragments were visualised using a UV transilluminator.

2.4 PCR AMPLIFICATION

2.4.1 Amplification from genomic DNA

PCR amplification of desired genes or DNA fragments from genomic DNA was carried out using 1 μmole each of forward and reverse primer **(Table 2.3)**, 100 μM dNTPs, and 1U Taq DNA Polymerase in the Taq Buffer provided (BRIT, Jonaki, Hyderabad, India). The mix was quickly denatured at 94°C for 4 min followed by 30 amplification cycles, each comprising of consecutive steps of (i) denaturation at 94°C for 45 sec, followed by (ii) annealing at 50°C-55°C for 45 sec to 1 min (depending on the T_m of the primers used), and (iii) extension at 72°C for varying time depending on the length of fragment to be amplified (approximately 1 min extension was used for amplifying 1 kb DNA fragment). Finally an extension step at 72°C for 10 min was carried out and the products were stored at 4°C till further use.

2.4.2 Reverse Transcriptase (RT) PCR

To assess if the isolated RNA carries any DNA contamination, PCR was carried out with total RNA using specific gene primers and Taq DNA polymerase. Only if no amplicons were obtained, the RNA was used for RT-PCR. Using total RNA (0.5 μg) as a template with gene specific primers **(Table 2.3)** and one-step RT-PCR enzyme mix (Qiagen). The master mix was incubated first for reverse transcriptase at 50°C for 30 min lead to generation of c-DNA, followed by 30 cycles of normal PCR (denaturation at 94°C

for 15 min, denaturation at 94°C for 45 sec, annealing at 52°C for gene specific primers of *ssb1* and *ssb2* and 54°C for *ssb3*, elongation at 72°C and following final extension at 72°C for 10 min). Amplification of genomic DNA with Taq DNA polymerase for each set of gene primers was kept as positive control.

Table 2.3 List of Primers used in this Study:

(A) For cloning of *ssb* gene and RT-PCR studies

Primers	Nucleotide Sequence	Restriction site
*ssb1*Fwd	5' GGCCATATGAGCATTAACATTGTC 3'	*Nde*I
*ssb1*Rev	5' GGCGGATCCTTAAAAATTTTCTGGTGC 3'	*Bam*HI
*ssb2*Fwd	5' GGCCATATGAACTATATCAACAAA 3'	*Nde*I
*ssb2*Rev	5' GGCGGATCCCTAGAAATTTGCGTTAGC 3'	*Bam*HI
*ssb3*Fwd	5' GGCCATATGAACAGCTGTGTTTTA 3'	*Nde*I
*ssb3*Rev	5' GGCGGATCCTAAAATGGAATATCGTC 3'	*Bam*HI
*all4780*Fwd	5' CCCTATATTACAATGTTGCCTC 3'	-
*all4778*Rev	5' TCGCCACCCCGGTATAGTTGCA 3'	-

(B) For generation of *ssb* promoter constructs

Primers	Nucleotide Sequence	Restriction site
P_{ssb1}Fwd	5' GCGCCGGAGCTCGGTATTTGCTGTACCGAG 3'	*Sac*I
P_{ssb1}Rev	5' GATCGGTACCCTGCCTTATCCTTTAGTACATAAGTACT 3'	*Kpn*I
P_{ssb2}Fwd	5' GCAACGAGCTCCAACAGATGTTTCTCCT 3'	*Sac*I
P_{ssb2}Rev	5' GCCCGGGTACCAACGCGAGAATTGAT 3'	*Kpn*I

P_{ssb3}Fwd	5’ GCAACGAGCTCTGTCAATAAAGTGGT 3’	*Sac*I
P_{ssb3s}Fwd	5' GAAGAGCTCGCCAACTATTAAATATTAAGTAT 3'	*Sac*I
P_{ssb3}Rev	5’ GCCCGGGTACCTAAATCCTCCTGACTGTA 3’	*Kpn*I

(C) For generating mutants:

Primers	Nucleotide Sequence	Restriction site
*ssb1*mut1Fwd	5’ CGTCCCCAGGAATTCGTACTG 3’	*Eco*RI
*ssb1*mut2Rev	5’ CTCATCTGCCGGATCCTTTAGTAC 3’	*Bam*HI
*ssb1*mut3Fwd	5’ GGTCAGTTGTTAGGATCCAAG 3’	*Bam*HI
*ssb1*mut4Rev	5’ GATTTTACTTGGAGCTCAGCGCTG 3’	*Sac*I
*ssb2*mut1Fwd	5’ GCCCCGAATTCAACGTTCGTGTT 3’	*Eco*RI
*ssb2*mut2Rev	5’ CTTTTAGGATCCATCATTTTGACG 3’	*Bam*HI
*ssb2*mut3Fwd	5’ GAAAATTACGGATCCCCTATG 3’	*Bam*HI
*ssb2*mut4Rev	5’ CATTTGAGAGCTCAAACTTGAG 3’	*Sac*I
*ssb3*mut1Fwd	5’ GAGGGAGGAATTCGGCGT 3’	*Eco*RI
*ssb3*mut2Rev	5’ GAAAATAGGGATCCGGGAAG 3’	*Bam*HI
*ssb3*mut3Fwd	5’ GGGTGTGAGGGATCCAAAG 3’	*Bam*HI
*ssb3*mut4Rev	5’ CTCGCAAGAGCTCAATAATTGTC 3’	*Sac*I

(D) For Electrophoretic Mobility Shift Assay (EMSA)

Primers	Nucleotide Sequence
*ssb1*upFwd	5’ GCGCCGGAGCTCCTGCATATTTTTATACTGT 3’
*ssb1*proRev	5’ GATCGGTACCCTGCCTTATCCTTTAGTACATAAGTACT 3’

*ssb1*upM1Rev	*5'* CTGCCTTATCCTTTA**CATG**ATAAGTACT 3'
*ssb1*upM2Rev	5' CTGCCTTATCCTTTAGTACATAA**CCTA**T3'
*ssb1*upM3Rev	5' CTGCCTTATCCTTTAGTAC**GAAC**GTACT 3'
35-mer Oligo	5' GACTGGTACAGTATCAGGCGCTGACCCACAACATC 3'

2.5 GENERATION OF RECOMBINANT BACTERIAL STRAINS

The different plasmids used in this study for generation of recombinant bacterial strains are listed in **Table 2.4.**

Table 2.4 List of plasmids used in this study

Plasmid	**Characteristics**	**Source**
pBluescript (pBS)	Cb^r, multicopy plasmid with MCS and ColE1 origin of replication	Stratagene
pET16b	Cb^r, Expression vector with N-terminal His-tag	Novagen
pFPN	Cb^r, Kan^r, Integrative expression vector	(172)
pAM1956	Kan^r, promoterless vector with g*fpmut2* reporter gene	(178)
pET*ssb1*	Cb^r, 0.35 kb *ssb1* gene cloned in pET16b at *Nde*I, *Bam*HI restriction sites	This study
pET*ssb2*	Cb^r, 0.38 kb *ssb2* gene cloned in pET16b at *Nde*I, *Bam*HI restriction sites	This study
pET*ssb3*	Cb^r, 0.55 kb *ssb3* gene cloned in pET16b at *Nde*I, *Bam*HI restriction sites	This study

pFPN*ssb1*	Cb^r, Kan^r, 0.8 kb *ssb1* cloned in pFPN at *Nde*I, *Bam*HI restriction sites	This study
pFPN*ssb2*	Cb^r, Kan^r, 0.83 kb *ssb2* cloned in pFPN at *Nde*I, *Bam*HI restriction sites	This study
pFPN*ssb3*	Cb^r, Kan^r, 1 kb *ssb3* cloned in pFPN at *Nde*I, *Bam*HI restriction sites	This study
pAM*ssb1*	1.1 kb *Xma*I-*Sal*I fragment from pFPN*ssb1* cloned in pAM1956 vector	This study
pAM*ssb2*	1.17 kb *Xma*I-*Sal*I fragment from pFPN*ssb2* cloned in pAM1956 vector	This study
pAM*ssb3*	1.3 kb *Xma*I-*Sal*I fragment from pFPN*ssb3* cloned in pAM1956 vector	This study

2.5.1 Preparation of competent cells of *E. coli* and transformation of DNA

Overnight grown *E. coli* cells were freshly inoculated in LB medium at a dilution of 1:100 and allowed to grow at 37°C with shaking at 140 rpm for 2 h or until the culture reached an OD_{600} of 0.8-1.0 per mL. This culture was then chilled on ice followed by centrifugation at 3000 rpm at 4°C for 5 min. The cell pellet was gently resuspended in Solution A (10 mM MOPS, pH 7, 10 mM RbCl) and incubated on ice for 5 min followed by centrifugation at 3000 rpm for 5 min at 4°C. The pellet was resuspended in Solution B (100 mM MOPS, pH 6.5, 50 mM $CaCl_2$, 10 mM RbCl) and incubated further on ice for 30-45 min. Cells were centrifuged and the pellet resuspended in Solution B containing

15% glycerol and either used immediately for transformation, or snap frozen in liquid nitrogen and stored at -70°C for future use.

Plasmid DNA (10-20 ng) or ligation mixture (containing up to 500 ng of DNA) was added to the transformation-competent *E. coli* cells and incubated on ice for 1 h. This was followed by a brief heat shock at 42°C for 90-120 sec. Cells were then quickly chilled on ice. Cells were allowed to recover at 37°C with shaking at 140 rpm for 1 h after addition of 1 mL LB medium. For plasmid DNA transformation, transformants were selected by plating 100 μL of the recovered cells. For transformation of ligation mix, the 1 mL recovered culture was harvested by centrifugation, resuspended in 100 μL of LB and plated on LB agar plates containing appropriate antibiotics. The different *E. coli* strains harbouring various plasmids are listed in **Table 2.5**.

2.5.2 Electroporation of plasmid DNA into *Anabaena* 7120

Three-day-old culture of *Anabaena* 7120 was concentrated to 10 μg chl*a* density mL^{-1} and washed four times with sterile stage II water. Plasmid DNA (1 μg) was added to 200 μL of the concentrated *Anabaena* 7120 cells and incubated under illumination for 1 h followed by electroporation at 1200 V with 5 msec pulse. BG-11, N^+ (2X) liquid media was added to the electroporated cells and incubated under light for 18 h. The cultures were then spotted on membrane and placed on BG-11, N^+, Neo_{25} plates under illumination. The membrane was transferred to fresh plates every 3-4 days.

2.5.3 Triparental conjugation in *Anabaena* 7120

Introduction of plasmid DNA into *Anabaena* 7120 by triparental conjugation was carried out in the following three steps:

(I) *E. coli* HB101 strain harbouring conjugal plasmid (pRL443) was conjugated with HB101 strain carrying pRL623 plasmid which codes for *Ava*I, *Ava*II and *Ava*III methylase.

(II) The next step involved conjugation of HB101 strain, obtained in step one, with HB101 strain carrying conjugable plasmid carrying gene of interest. The resultant recombinant *E. coli* strain will have three plasmids, namely the cargo plasmid, pRL623 and pRL443 and is now ready to be conjugated in *Anabaena* 7120. Also the DNA will be methylated which would protect it from endonucleolytic activities of *Ava* enzymes in *Anabaena* 7120.

(III) The final step is conjugation of recombinant HB101 strain harbouring the three plasmids obtained in step two with wild type *Anabaena* 7120. The conjugal plasmid (pRL443) aids in transfer of methylated cargo plasmid to recipient *Anabaena* strain.

For conjugation between *E. coli* strains, mid-log phase *E. coli* cultures were washed with LB medium without any antibiotic and then mixed in a centrifuge tube followed by incubation at 37℃ for 3 h to allow conjugation. After 3 h, these cells were centrifuged at 3,000 rpm for 1 min and spread on solid medium containing appropriate antibiotic and incubated overnight at 37℃. Recombinant colonies obtained were inoculated in LB medium for growth with appropriate antibiotics.

For conjugation between *E. coli* and *Anabaena* 7120 strains, mid-log phase cultures (~3 h for *E. coli* and 3 d for *Anabaena*) were washed with respective media

without any antibiotic and then mixed in a glass tube followed by incubation at 27°C for 3 h under constant illumination to allow conjugation. After 3 h the settled cells were spread on nitrocellulose membranes placed on solid medium without any antibiotic and incubated O/N at 27°C under illumination. After 24 h the membranes were transferred on to fresh solid medium containing appropriate antibiotics to facilitate selection of recombinant *Anabaena* colonies and the membranes were transferred on to fresh solid media after every three days. Recombinant *Anabaena* colonies were then inoculated in liquid medium for further analysis. Recombinant *Anabaena* strains thus obtained were subcultured on every third day to achieve faster and better segregation and subsequently the strains were maintained in liquid media containing appropriate antibiotics and were subcultured every week. The wild type and different recombinant *Anabaena* strains used in this study are listed in **Table 2.5**.

Table 2.5 Bacterial strains used in this study

Strain	Characteristics	Source/Reference
***E. coli* strains**		
DH5α	F^- *recA*41 *endA*1 *gyrA*96 *thi*-1 *hsdR*17 (rk^- mk^-) *supE*44 *relA*λ Δ*lacU*169	Lab collection
BL21(DE3)(p*lysS*)	F^- *ompT gal dcm lon hsd*S_B(r_B^- m_B^-) λ(DE3) p*lysS*(Cm^r)	Novagen
HB101	F^-mc^r Bm^r rhsdS20($r_B^-m_B^-$) *recA*13 *leu* B6ara-14 *pro*A2 *lac*Y1 *gal*K2 *xyl*-5 *mtl*-1	Lab collection

	*rpsL*20 (Sm^R) *gln*V44 λ^-	
DH5α(pET*ssb1*)	Cb^r, DH5α strain harbouring pET*ssb1* plasmid	This study
DH5α(pET*ssb2*)	Cb^r, DH5α strain harbouring pET*ssb2* plasmid	This study
DH5α(pET*ssb3*)	Cb^r, DH5α strain harbouring pET*ssb3* plasmid	This study
BL21(pLysS) (pET*ssb1*)	Cm^r, Cb^r, *E. coli* BL-21 cells harbouring the plasmid pET*ssb1*	This study
BL21(pLysS) (pET*ssb2*)	Cm^r, Cb^r, *E. coli* BL-21 cells harbouring the plasmid pET*ssb2*	This study
BL21(pLysS) (pET*ssb3*)	Cm^r, Cb^r, *E. coli* BL-21 cells harbouring the plasmid pET*ssb3*	This study
***Anabaena* strains**		
Anabaena sp. strain PCC7120 (*Anabaena* 7120)	Fresh water, filamentous, heterocystous, diazotroph	Lab collection
AnpAM	Nm^r, *Anabaena* 7120 harbouring the plasmid pAM1956	(179)
An*ssb1*$^-$	Nm^r, *Anabaena* 7120 with deletion of *ssb1* gene	This study
An*ssb2*$^-$	Nm^r, *Anabaena* 7120 with deletion of *ssb2*	This study

	gene	
An*ssb3*$^{-}$	Nm^{r}, *Anabaena* 7120 with deletion of *ssb3* gene	This study
An*ssb1*$^{+}$	Nm^{r}, *Anabaena* 7120 harbouring the plasmid pAM*ssb1*	This study
An*ssb2*$^{+}$	Nm^{r}, *Anabaena* 7120 harbouring the plasmid pAM*ssb2*	This study
An*ssb3*$^{+}$	Nm^{r}, *Anabaena* 7120 harbouring the plasmid pAM*ssb3*	This study
AnP_{ssb1}*:gfp*	Nm^{r}, *Anabaena* harbouring the plasmid pAMP_{ssb1}*:gfpmutII*	This study
AnP_{ssb2}*:gfp*	Nm^{r}, *Anabaena* harbouring the plasmid pAMP_{ssb2}*:gfpmutII*	This study
AnP_{ssb3}*:gfp*	Nm^{r}, *Anabaena* harbouring the plasmid pAMP_{ssb3}*:gfpmutII*	This study
AnP_{ssb3s}*:gfp*	Nm^{r}, *Anabaena* harbouring the plasmid pAMP_{ssb3s}*:gfpmutII*	This study

2.6 EXTRACTION, PURIFICATION AND DETECTION OF CELLULAR PROTEINS

2.6.1 Extraction of proteins under denaturing conditions, estimation of protein content and gel electrophoresis

Total cellular protein from either *E. coli* or *Anabaena* was extracted from harvested cells by resuspending the pellet in minimum amount of water followed by addition of equal amount of 2X Laemmli's buffer (180) **(Table 2.6)** and boiling for 10 min. The supernatant obtained after centrifugation at 10,000 rpm for 10 min contained the total cell free protein extract.

Protein estimation was carried out by Lowry's method (181). For estimating the protein concentration, 5 µL of the extracted protein was diluted to a final volume of 500 µL with D/W. Protein was solubilised by the addition of 50 µL of 10% deoxycholate (DOC), followed by mixing and incubation at 25°C for 10 min. Solubilised protein was precipitated by the addition of 50 µL of 78% Tri-chloroacetic acid (TCA). The precipitated protein was centrifuged at 10000 rpm for 10 min. The pellet was resuspended in 500 µL of D/W, followed by the addition of 500 µL of Lowry's Reagent and incubated for 15 min at room temperature. The reaction was developed by the addition of 250 µL of Folin-Ciocalteau's reagent and incubation at room temperature in dark for 30 min. The colour was estimated spectrophotometrically at 750 nm. Different concentrations of Bovine Serum Albumin (BSA) (Sigma Aldrich, USA) were used to plot a standard graph.

2.6.2 Overexpression and Purification of recombinant proteins

Overnight grown *E. coli* BL21(p*lysS*)(DE3) cultures harbouring different plasmids were inoculated in LB Cm_{34} Cb_{100} at a dilution of 1:100 and incubated at 37°C with orbital shaking at 140 rpm until the culture reached an OD_{600} = 1 per mL. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM to these cultures and incubation continued for a period of 3 h. The cells were then harvested by centrifugation at 5000 rpm for 5 min and pellet was snap frozen in liquid nitrogen followed by storage at -70 °C till further use.

The cell pellets were resuspended in Ni-NTA binding buffer approximately one tenth volume of the induced culture volume. Cells were lysed by sonication on ice. Sonication was carried out in Branson Digital Sonifier at 35% amplitude, pulse ON 1 sec, pulse OFF 2 sec, for 2 min. The lysate was clarified by centrifugation at 10000 rpm for 30 min at 4°C. The clarified lysate was kept for binding to Ni-NTA resin (1 mL Ni-NTA slurry per 400 mL induced culture volume) overnight at 4°C with constant shaking at 50 rpm. The Ni-NTA-lysate suspension was then passed through a column open from top, perforated at the bottom and layered with glass wool. The column was washed with Ni-NTA wash buffer sequentially from lower to higher imidazole concentrations. All eluates were collected for analysis by SDS-PAGE. The aliquots containing purified protein were transferred to a dialysis membrane and dialysed against dialysis buffer overnight at 4°C. Dialysed protein was aliquoted and stored at -70°C till further use.

The protein extracts were separated by electrophoresis on a denaturing 12% SDS-polyacrylamide gel at 100 V for 1h followed by 200 V till the dye front reached the end of the gel. The complete protein profile was visualized by staining the gel with (CBB) G-

250 for 30 min to 1 h followed by destaining with Destaining Solution (DS)-I for 10 min and DS-II for 1 h to overnight.

Oligomeric status of purified SSB proteins was determined by Size exclusion chromatography which was performed at room temperature using HPLC system (Pharmacia Amersham) equipped with Superdex-200 HR 10/30 column. The column was equilibrated with at least three bed volumes of 100 mM Tris-HCl (pH 8.0), 500 mM NaCl prior to each run. A typical flow rate of 0.6 mL min^{-1} was maintained. Absorbance was measured at 220 nm to monitor elution of proteins from the column.

Table 2.6 List of reagents used for protein extraction, resolution and estimation

Reagent	Composition
2X Laemmli's Buffer	0.5 M Tris-HCl, pH 6.8, 5% (w/v) 2-mercaptoethanol, 2% (w/v) SDS, 20% Glycerol, 0.1% Bromophenol Blue (BPB), 0.076% EGTA, 0.2% Sodium azide, 0.02% PMSF
Running Buffer	0.3% Trizma Base, 1.44% Glycine, 0.1% SDS
Destaining Solution (DS)	DS-I: 10% Glacial acetic acid, 50% Methanol DS-II: 10% Glacial acetic acid, 10% Methanol, 2 % Glycerol
Coomassie Brilliant Blue (CBB) G250	0.2% Coomassie Brilliant Blue G 250, 20% Glacial acetic acid, 40% Methanol
Transfer Buffer	0.125 M Trizma Base, 0.192 M Glycine
Tris Saline Buffer	0.05 M Trizma Base, 0.9% NaCl
Maleic Acid (MaNa) Buffer	0.1 M Maleic acid, 0.15 M NaCl

Reaction Buffer	0.1 M Trizma Base, 0.1 M NaCl
Ni-NTA	Nickel-Nitrilo tri-acetate (Ni-NTA) slurry Qiagen, Germany
Ni-NTA binding buffer	0.1 M Tris pH 8, 0.3 M NaCl, 10 mM Imidazole
Ni-NTA wash buffer	Ni-NTA binding buffer containing varying concentrations of imidazole from 50 mM to 200 mM with 10 mM increments
Dialysis and Storage buffer	0.1 M Tris pH 8, 0.3 M NaCl, 25% Glycerol
1X Blocking Reagent	1% Blocking Reagent (Roche, Germany) in 1X MaNa Buffer
NBT/BCIP	NBT/BCIP (Roche, Germany) diluted 1:50 in 1X Reaction Buffer

2.6.3 Western blotting and immunodetection

For identification of specific proteins, the gel was equilibrated in transfer buffer for 1 h at ambient temperature. Proteins from the gel were transferred on to the nitrocellulose membrane by electroblotting at a constant current of 0.4 Amp for 3 h. Blot was washed in Tris Saline buffer (TSB) containing 1% blocking reagent for 1 h. This blot was probed with primary antibody appropriately diluted in 1% blocking reagent in TSB for 1 h. The blot was then washed three times with Maleic Acid Buffer for 20 min each and then probed for 1 h with 1:10000 diluted secondary antibody (anti-rabbit IgG raised in sheep linked to alkaline phosphatase) Roche Diagnostics, GmBH. The blot was then washed with Maleic Acid buffer three times for 20 min each, rinsed in reaction buffer and colour development carried out using NBT/BCIP.

2.6.4 Glutaraldehyde cross-linking

The purified recombinant native proteins (50 µg) were individually cross-linked using glutaraldehyde (20 µl of 25% w/v) in the absence or presence of M13 ssDNA in cross-link buffer at room temperature for two minutes. Freshly prepared 25 µL $NaBH_4$ (2 M in 0.1 M NaOH) was added and incubated at room temperature for twenty minutes, followed by precipitation of protein with 10% Na-deoxycholate, then with 22.5 µl of 78% TCA, followed by cold acetone. The pellet was air dried, solubilised in 1X Laemmli's buffer by heating at 80°C for 10 min, separated by SDS-PAGE and visualized by staining with CBB G-250.

2.7 DNA-PROTEIN INTERACTIONS ASSAYS

2.7.1 Electrophoretic Mobility Shift Assay (EMSA)

Single-stranded oligonucleotide (10 ng) was end-labelled with γ-^{32}P-ATP using Polynucleotide Kinase. The labelled oligo was incubated in the presence of specified concentrations of the SSB1, SSB2 and SSB3 proteins in binding buffer [20 mM Tris-HCl, pH 8, 1 mM $MgCl_2$, 100 mM KCl, 8 mM Dithiothreitol (DTT), 4% sucrose, 80 µg mL^{-1} Bovine Serum Albumin (BSA)] for 30 min at room temperature and electrophoretically separated subsequently on 12% non-denaturing polyacrylamide gel at 150 V for 2 h in 1X Tris-borate EDTA (TBE) buffer. Imaging of the radioactive gel was carried out using Phosphor imager Typhoon Trio Variable mode imager (Wipro-GE-HealthCare, USA).

2.7.2 Fluorescence quenching

All proteins have an inherent pattern of fluorescence emission when excited at a specific wavelength due to the presence of aromatic amino acids such as tryptophan, tyrosine and phenylalanine. This property was used to test the binding efficiency of proteins to DNA, since upon binding at saturating concentrations of DNA the tryptophan-based emission of the protein would decrease (182).

All the three SSB proteins were first checked for their characteristic wavelength for excitation and emission in the binding buffer (20 mM Tris-HCl pH 8.0, 1 mM EDTA) containing three different concentrations of NaCl (0, 20 and 100 mM). The excitation wavelength for the three SSB proteins was found to be 282 nm, while the emission wavelength varied slightly, being 310 nm, 340 nm and 335 nm for SSB1, SSB2 and SSB3 proteins respectively. The change in the intensity of the emitted fluorescence was measured in the presence of increasing concentration of ssDNA [poly(dT) or M13 ssDNA]. The graph of relative fluorescence (%) as a function of poly(dT) concentration was used to determine the binding constant for the individual proteins to ssDNA as described earlier (183). The binding constants were calculated as the reciprocal of the concentration of poly(dT) at which 50% fluorescence compared to the initial 100% was detected. The graph depicting quenching expressed as the ratio of difference in fluorescence to initial fluorescence ($\Delta F/F_i$) as a function of the ratio of concentrations of poly(dT) and protein was used to determine the binding modes or occlusion site of the proteins as described earlier (41). It corresponded to the $[nt]_{poly(dT)}$/[Protein] value at the point of saturation of quenching of fluorescence. During titration, solutions were added from concentrated samples and correction for dilution was made as required. All

fluorescence measurements were performed with Jasco spectrofluorimeter FP6500 (Japan) using a quartz cuvette of 1 cm path length at room temperature. Fluorescence of SSB in the absence of ssDNA was considered as 100% (initial fluorescence Fi). Relative fluorescence calculated as % of ratio of fluorescence in the presence of ssDNA (F_s) and initial fluorescence (F_s/F_i). Reciprocal of poly(dT) concentration which results in 50% relative fluorescence is the binding affinity of the SSB for ssDNA.

2.8 BRIGHT-FIELD AND FLUORESCENCE MICROSCOPY

E. coli and *Anabaena* cultures were spotted on glass slides and observed under bright field to study phenotype using 40 X objective (600 X magnification). For qualitative analysis of GFP fluorescence, the cells were exposed to an excitation wavelength of 480 nm (λ_{ex} 480) and the fluorescence was captured at a wavelength of 520 nm (λ_{em} 520). This wavelength was specifically absorbed by the blue pigment phycocyanin which transfers the energy to chlorophyll *a*, which shows a typical red autofluorescence (λ_{em} 710). Microscopy was carried out using a Carl Zeiss Axioscop 40 microscope and pictures were captured with the help of a charge-coupled device (CCD) axiocam MRc (Zeiss) camera attached to the microscope.

2.9 FLUORIMETRIC ANALYSIS OF GFP EXPRESSION

The expression of GFP in *E. coli* or *Anabaena* cells was quantitated using a Jasco FP 1500 spectrofluorimeter. The cells were exposed to an excitation wavelength of 490 nm (λ_{ex} 490) and the emission of fluorescence was recorded at an emission wavelength of 510 nm (λ_{em} 510) under normal growth conditions and during stress period, along with

appropriate controls. The activity of promoter was calculated using Fluorescing units (GFP arbitrary units) per µg chlorophyll *a*.

CHAPTER 3

Bioinformatic and Biochemical analysis of ***Anabaena*** **SSB** *genes/proteins*

3.1 INTRODUCTION

The *ssb* gene coding for single stranded DNA binding (SSB) protein is present across bacterial genomes but differ in their gene organization. The first Bioinformatic analysis on *ssb* genes in bacterial genomes was reported in 2004, wherein 87 bacterial genomes were screened for *ssbA* homologues for its organization in the genome (105). The study revealed that in about 15 bacteria, mostly Gram negative such as *E. coli*, *ssb* is placed adjacent to but divergent from *uvrA* gene, while 35 genomes mostly of Gram positive bacteria had positioning similar to that of *ssbA* of *Bacillus subtilis*, wherein the *ssbA* gene is positioned between genes for ribosomal proteins, *rpsF* and *rprR* (105). The positioning of *ssbA* gene between ribosomal protein genes is also preserved in the representatives of *Thermotogales* and *Thermus/Deinococcus* group, and Gram-negative species from the phyla *Spirochaetales*, *Aquificales*, *Chlorobi* and ε subdivision of proteobacteria. Other subdivisions of proteobacteria as well as cyanobacteria, fusobacteria did not display either of the two types of *ssb* gene organisation (105).

Phylogenetic analyses of SSB proteins based on amino acid sequence suggest that SSB superfamily is possibly derived from a common ancestral domain present in the genome at the very base of the evolutionary tree (184). The different SSB families are grouped under eight distinct phylogeny groups which included eukaryotes, crenarchaea, euryarchae, mitochondria, Gram negative bacteria, Gram positive bacteria, phages and SsbB in genetic islands in proteobacteria (184). The genetic islands, which correspond to conserved genetic cluster, contain genes encoding DNA-processing enzymes: ParA, ParB, and TopB topoisomerase and other conserved hypothetical proteins.

SSB proteins, in general, are 170-180 amino acids long and are characterised by an N-terminal OB-fold and a C-terminal acidic tail separated by P/G rich spacer (6, 39). Some of the naturally competent bacteria such as *B. subtiilis*, additionally have an alternate SSB which is about ~117 amino acids long (105), lack the C-terminal tail as well as the P/G-rich region, but functions as SSB and is involved in competence development (106, 107) and these are classified in Group III. These are the smallest reported bacterial SSB proteins. Only the thermophilic bacteria, *Thermotoga maritima* and *T. neapolitana* have a single full length SSB, which is comparatively smaller i.e. about 141 amino acids long compared to other bacterial SSBs (185).

3.2 RESULTS

3.2.1 *In silico* analysis of *Anabaena ssb* genes and encoded proteins

Two ORFs '*alr0088'* and '*alr7579'* of *Anabaena* 7120 are annotated as coding for SSB-like proteins in the cyanobacterial database (http://genome.microbedb.jp/cyanobase) and have been designated as SSB1 and SSB2 respectively **(Fig 3.1)**. Conserved Domain Database (CDD) analysis of these two proteins revealed that the protein sequence is terminated immediately after the N-terminal domain which contains a ssDNA binding region corresponding to the conserved oligonucleotide-oligosaccharide fold (OB-fold) **(Fig. 3.1)**. They lack the P/G-rich spacer as well as the C-terminal tail rich in acidic amino acids **(Fig. 3.1)**. However, a third ORF encoded by '*all4779*' and annotated as a 'hypothetical protein' in cyanobase, was found to have all three regions typical of bacterial SSBs **(Fig. 3.1)**, and was designated as SSB3. The length of the N-terminal

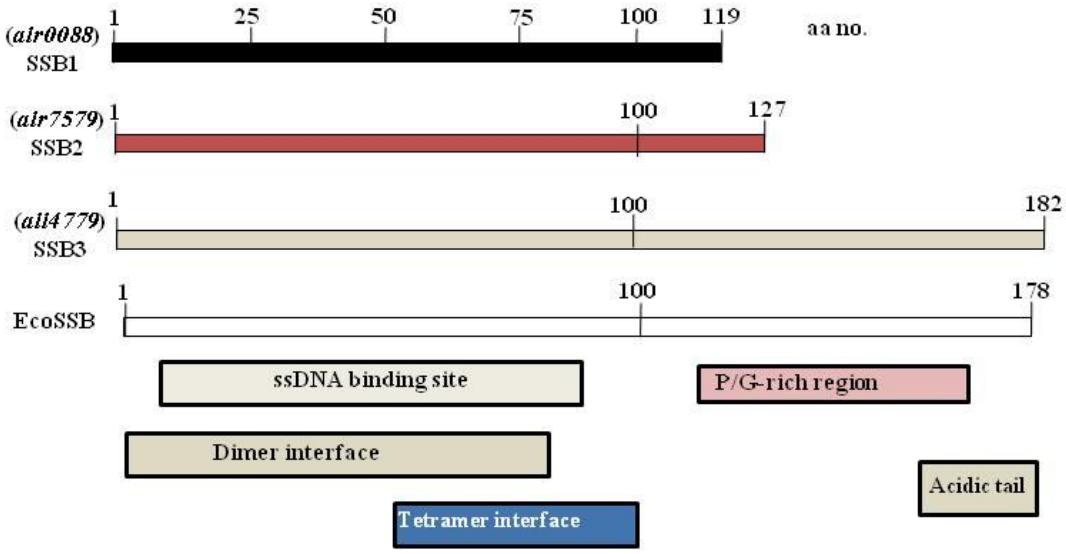

Fig. 3.1 Domain organisation of *Anabaena* 7120 SSB proteins.

Conserved Domain Database (CDD) analysis of *Anabaena* Alr0088 (SSB1), Alr7579 (SSB2) and All4779 (SSB3) proteins and *E. coli* SSB (EcoSSB) protein. The OB-fold corresponding to ssDNA binding region, dimer and tetramer interfaces for all the proteins and the P/G-rich region and acidic tail in SSB3 and EcoSSB are indicated. The numbers on top of each horizontal bar indicate the amino acid position.

domain predicted by CDD for all three SSB-like proteins is similar to that of *E. coli* SSB **(Fig. 3.1)**. However, due to low and scattered identity of SSB3 at amino acid level with other bacterial SSBs, it has possibly not been annotated as a SSB protein in *Anabaena.* The homologos of all three putative *ssb*-like genes of *Anabaena* 7120 are present across cyanobacterial genome (http://genome.microbedb.jp/cyanobase).

The *alr0088* (*ssb1*), *alr7579* (*ssb2*) and *all4779* (*ssb3*) genes respectively encode 119, 127 and 182 amino acid long polypeptides **(Fig 3.2A)**, with an estimated molecular mass of 13, 14 and 20 kDa respectively. The three genes are scattered across different positions on the chromosome **(Fig 3.2A)**. Comparison of the amino acid sequences of the three *Anabaena* SSBs with other bacterial SSB proteins was carried out using BLAST-P software (http://www.ncbi.nlm.nih.gov/blast). Both SSB1 and SSB2 exhibited about 57-58% similarity and 35-37% identity compared to *E. coli* SSB, while SSB3 exhibited 47% similarity and 26% identity with *E. coli* SSB **(Fig. 3.2B)**. The extent of similarity with SSB proteins of *D. radiodurans* and *B. subtilis* was about 49% and 54-61% respectively for SSB1 and SSB2 and 40-42% for SSB3 **(Fig. 3.2B)**. *Anabaena* SSB proteins exhibited 64-67% similarity with the corresponding homologues of the unicellular cyanobacterium, *Synechocystis* PCC6803 **(Fig. 3.2B)**.

The overall identity of SSB3 with EcoSSB was 26%, which included 21% identical and 18% similar residues at the N-terminus and a near identical C-terminal acidic tail **(Fig. 3.3)**. The intervening region corresponding to P/G rich spacer differed in the ratio of proline to glycine residues in the two proteins **(Fig. 3.3)**.This region in SSB3 is proline-rich (19 residues) and has only two glycine residues, as against EcoSSB which is glycine rich (21 residues) along with 8 prolines in the corresponding region **(Fig. 3.3)**.

(A)

Gene Annotation	Position on chromosome	Annotated as	Size	Designated as	DNA binding domain
alr0088	91483-91842	ssDNA binding protein	359 bp (119 aa)	*ssb1*	1- 62 aa
alr7579	74007-74390	ssDNA binding protein	383 bp (127 aa)	*ssb2*	1- 67 aa
all4779	5694331-5694879	Hypothetical protein	549 bp (182 aa)	*ssb3*	1-122 aa

(B)

	7120 SSB1	7120 SSB2	7120 SSB3	Syn SSB	E. coli SSB	*Deino* SSB	Bs SsbA	Bs SsbB
7120-SSB1	100%	67% (42%)	41% (22%)	77% (62%)	58% (35%)	49% (27%)	57% (36%)	54% (38%)
7120-SSB2	67% (42%)	100%	25% (16%)	65% (48%)	57% (37%)	49% (28%)	61% (30%)	56% (30%)
7120-SSB3	41% (22%)	25% (16%)	100%	64% (50%)	47% (26%)	40% (27%)	41% (22%)	42% (26%)

Fig. 3.2 Annotation of *Anabaena* 7120 SSB proteins and its comparison with other bacterial SSBs.

(A) The gene and protein annotation of the three *Anabaena* 7120 SSB proteins and the position of the genes on the chromosome as mentioned in the genome data base. The total size of the gene and corresponding protein, and the length and position of the DNA binding domain for the three proteins is also stated. (B) Percentage of amino acid sequence similarity and identity (given in parentheses) of the three *Anabaena* 7120 SSB proteins amongst themselves, the corresponding SSB of unicellular cyanobacterium, *Synechocystis* PCC6803 (Syn), *E. coli*, *Deinococcus radiodurans* (Deino) and SsbA and SsbB of *Bacillus subtilis.*

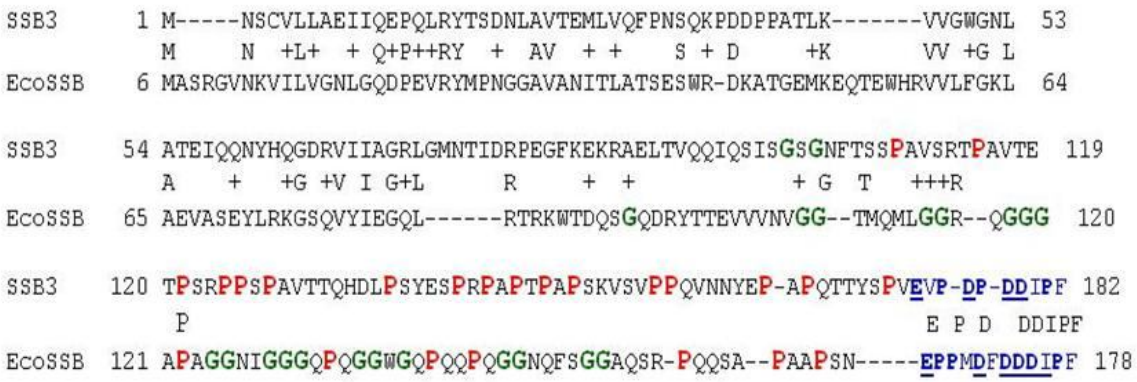

Fig. 3.3 Comparison of *Anabaena* 7120 SSB3 protein with *E. coli* SSB.

Comparison of homology between predicted amino acid sequence of SSB3 and EcoSSB. The identical amino acids are indicated by letters and similar amino acids with a '+' sign. The proline (P) and glycine (G) residues beyond the OB fold are shown in red and green respectively, while the acidic residues at the C-terminal end are in blue and underlined. The numbers on the left and right hand side correspond to amino acid residues.

A comparison of protein sequences of SSB from 24 bacterial species, which included both gram negative and gram positive bacteria, was carried out to generate a phylogenetic tree **(Fig. 3.4)**. Sequences of all three SSBs of *Anabaena* 7120 and two SSBs of *Synechocystis* 6803 were included among the cyanobacterial SSBs. The tree indicates three groups, with majority of the bacterial SSBs present in Group I. Group II consisted of SSB proteins of *Campylobacter jejuni* and *Helicobacter pylori* and Group III, SSB of *Thermotoga maritima* **(Fig. 3.4)**. Group I was further divided into two sub groups IA and IB. The truncated SSB proteins of *Anabaena* 7120 and the corresponding SSB of *Synechocystis* PCC6803 along with that of *Treponema pallidum* were part of sub group IB. SSB1 (Alr0088) of *Anabaena* 7120 and Slr0925, coding for SSB of *Synechocystis* 6803 were closely grouped **(Fig. 3.4)**. The SSB3 of *Anabaena* 7120 was closely grouped with Slr1034 of *Synechocystis* 6803 and closest to SSB of archaebacterium *Sulfolobus solfataricus* **(Fig. 3.4)**. This suggested that the truncated SSBs and the full length SSBs of *Anabaena* 7120 are not evolutionary linked.

3.2.2 Cloning, Expression and Purification of *Anabaena* SSB proteins from *E. coli*

3.2.2.1 Cloning of *ssb* genes

The ORFs corresponding to *ssb1* (*alr0088*), *ssb2* (*alr7579*) and *ssb3* (*all4779*) were individually PCR amplified as 0.36 kb, 0.38 kb and 0.55 kb DNA fragments respectively **(Fig. 3.5)** from *Anabaena* 7120 chromosomal DNA, using gene specific primers **(Table 2.3)**. The primers were designed on the basis of the available genome sequence of *Anabaena* 7120 (http://genome.microbedb.jp/cyanobase). No amplification

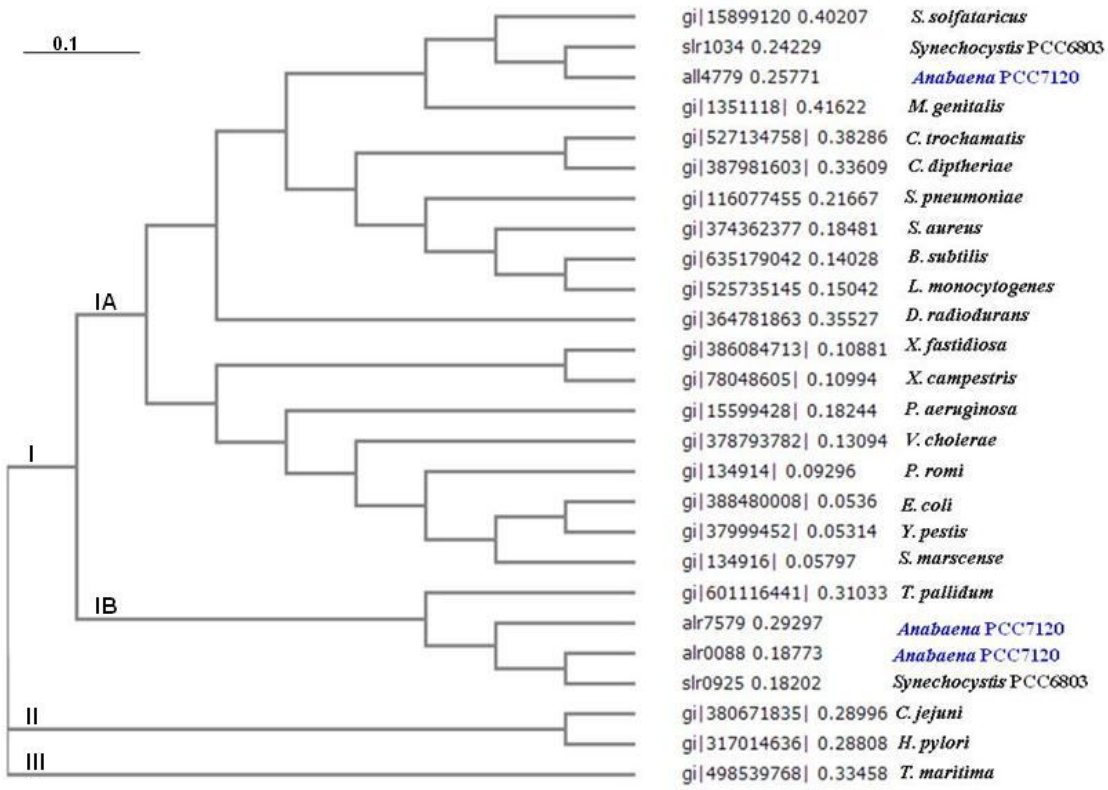

Fig. 3.4 Neighbour-joining tree of SSB proteins among bacterial genera.

Bacterial SSBs from 24 bacteria were used and included two SSBs from unicellular cyanobacterium, *Synechocystis* PCC6803 and three from the filamentous cyanobacterium, *Anabaena* PCC7120. Alignments and dendrogram was made with Clustal Omega with neighbour joining method. Bootstrap values in decimals are indicated at the end of branching points. The gene number or the accession number of each of the SSB protein used for analysis is also mentioned at the end of the corresponding branching point. The SSB proteins of *Anabaena* PCC7120 are highlighted in blue.

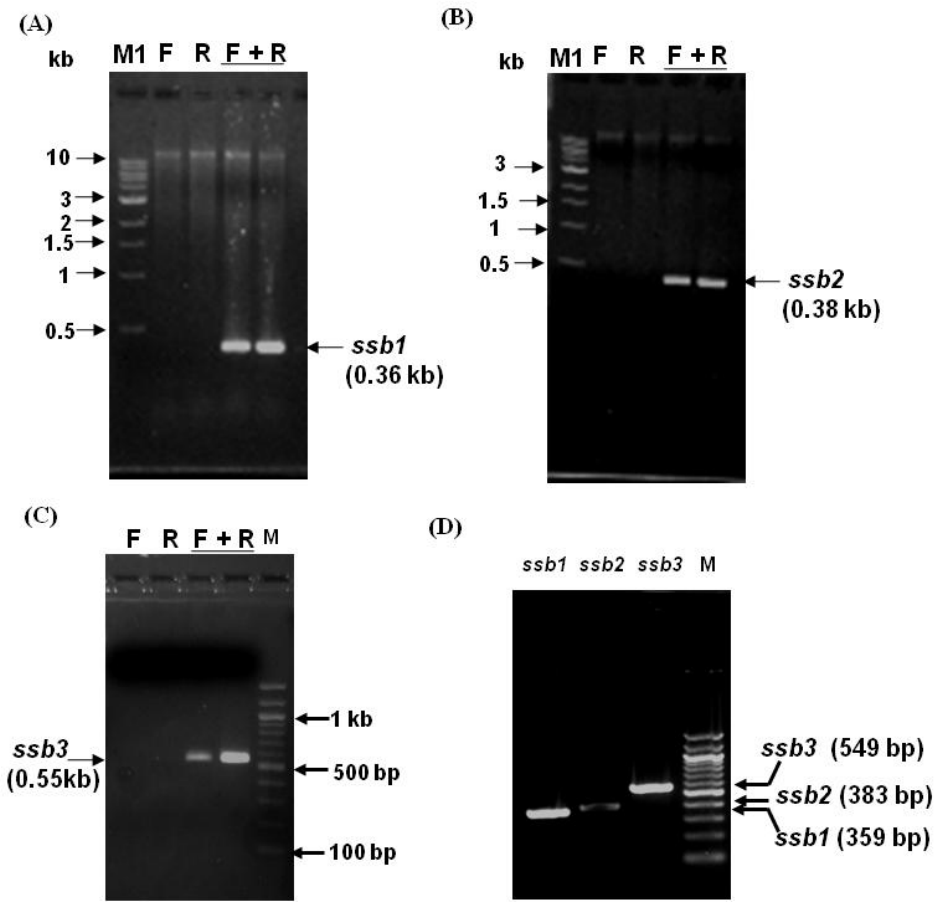

Fig. 3.5 PCR amplification of the three *ssb* genes of *Anabaena* 7120.

(A-C) *Anabaena* 7120 genomic DNA was used as a template for PCR amplification of (A) *ssb1*, (B) *ssb2*, and (C) *ssb3* genes using gene specific primers. F and R indicate forward and reverse primers respectively. (D) Purified amplicons. The different DNA fragments were separated electrophoretically on 0.8% agarose gel stained with ethidium bromide and visualised using a UV-transilluminator. The different amplicons along with their size have been indicated with arrows. Two different DNA markers were used, designated as M (100 bp ladder) and M1 (1 kb ladder). The molecular mass of the major DNA fragments in the DNA marker are indicated.

was observed when only one of the primers i.e. either forward or reverse was used during PCR amplification **(Fig. 3.5A-C)**, thus confirming that the amplification was specific for the set of primers used. These amplicons were digested with *Nde*I and *Bam*HI restriction endonucleases, ligated to the expression vector pET16b **(Table 2.4)** cut with the same set of restriction enzymes, transformed into *E. coli* DH5α **(Table 2.5)** and selected on LB Cb_{100} plates. Several of the Cb^r colonies for each set i.e. DH5α(pET*ssb1*), DH5α(pET*ssb2*) and DH5α(pET*ssb3*) were tested for the presence of insert by colony PCR using the corresponding gene-specific primers. Plasmid DNA from one colony of each set of the PCR positive colonies upon digestion with *Nde*I and *Bam*HI restriction enzymes released the 5.7 kb vector and corresponding inserts of ~0.36 kb, 0.38 kb and 0.55 kb **(Fig. 3.6)** respectively. The plasmids were correspondingly designated as pET*ssb1*, pET*ssb2* and pET*ssb3* **(Table 2.4)**. Nucleotide sequence of the cloned *ssb* inserts in pET*ssb1*, pET*ssb*2 and pET*ssb3* was ascertained by DNA sequencing. The nucleotide sequence has been submitted to GenBank (GenBank Accession No. GU225949, GU225950, GU225951) (Annexure I-III).

3.2.2.2 Overexpression and purification of *Anabaena* SSB proteins

The plasmids pET*ssb1*, pET*ssb2* and pET*ssb3* were individually transformed into *E. coli* BL21(p*lysS*) (DE3) cells **(Table 2.5)** and overexpression of the proteins induced by the addition of 1 mM IPTG to the corresponding log-phase cultures. The SSB1, SSB2 and SSB3 proteins were found to be maximally induced after 3 h of induction with IPTG **(Fig. 3.7 A-C)**. The overexpressed proteins were purified to near homogeneity using Ni-NTA affinity chromatography. The proteins were eluted under native conditions

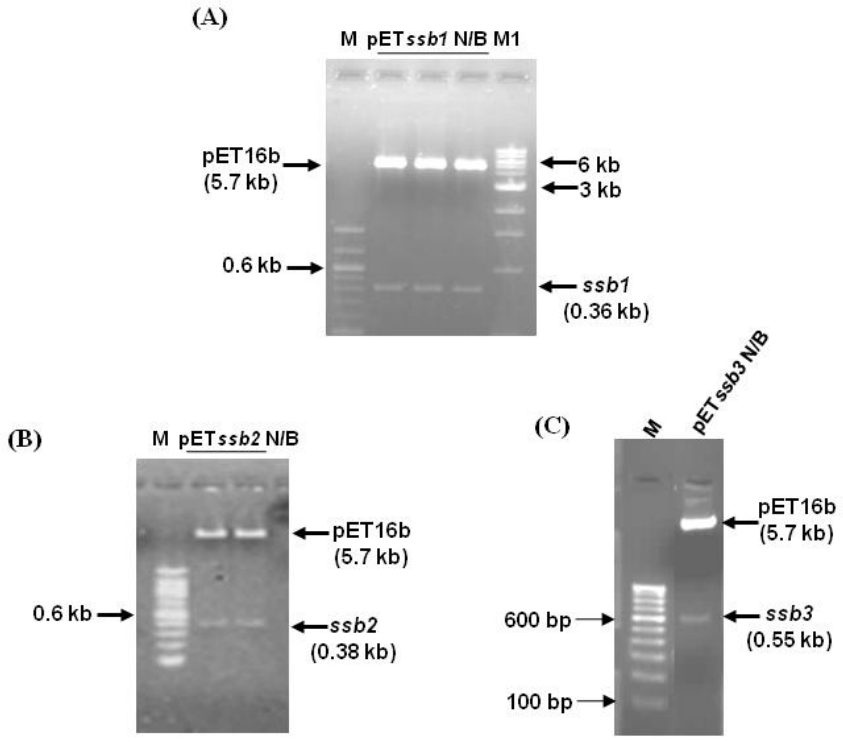

Fig. 3.6 Restriction digestion of cloned pET*ssb* plasmids.

Restriction digestion of (A) pET*ssb1*, (B) pET*ssb2*, and (C) pET*ssb3* with restriction endonucleases, *Nde*I (N) and *Bam*HI (B). The release of the vector DNA (5.7 kb pET16 b) and the corresponding inserts are indicated with arrows. Lanes M and M1 indicate DNA markers. Other details are as described in legend to Fig. 3.5.

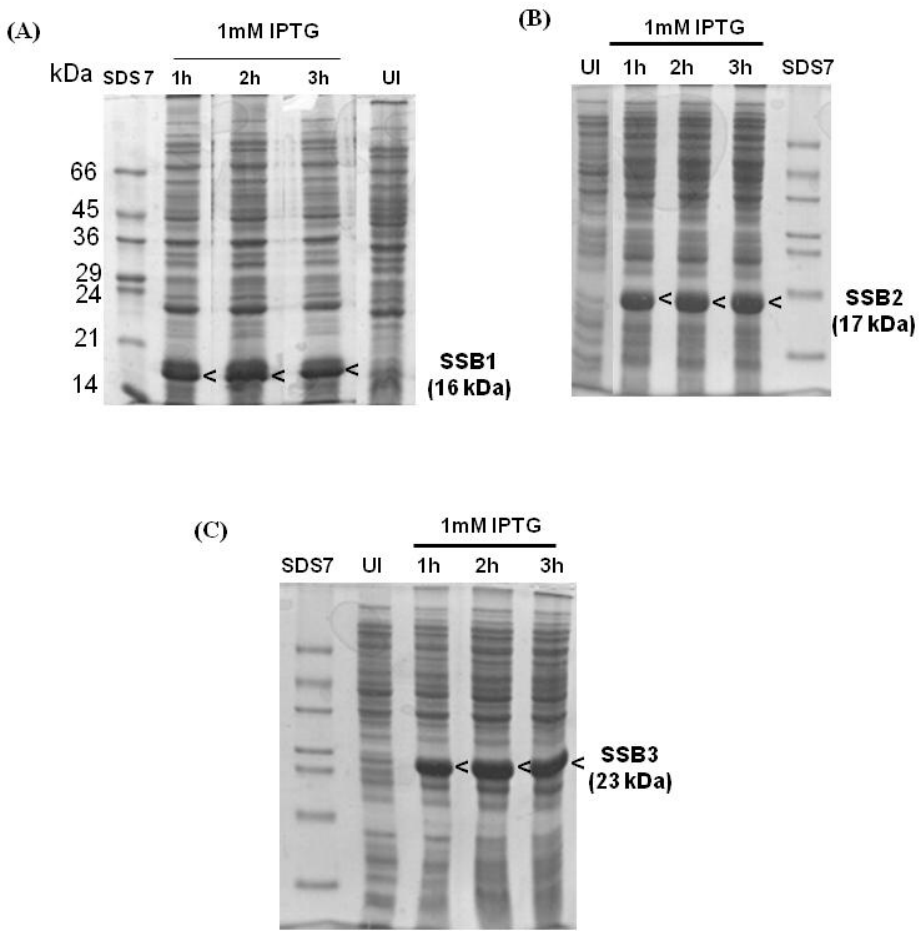

Fig. 3.7 Overexpression of *Anabaena* SSB proteins in *E. coli.*

Logarithmic phase *E. coli* BL21(p*lysS*)(DE3) cells harbouring (A) pET*ssb1*, (B) pET*ssb2*, and (C) pET*ssb3* plasmids were induced with 1 mM IPTG for up to 3 h and total protein extracted using Laemmli's buffer. Total protein extracts were resolved by 12% SDS-PAGE, followed by staining with Coomassie Brilliant Blue (CBB) dye. The three SSB proteins along with their apparent molecular mass are indicated. The molecular mass of the different protein bands in the protein marker (SDS-7) used is also indicated in (A).

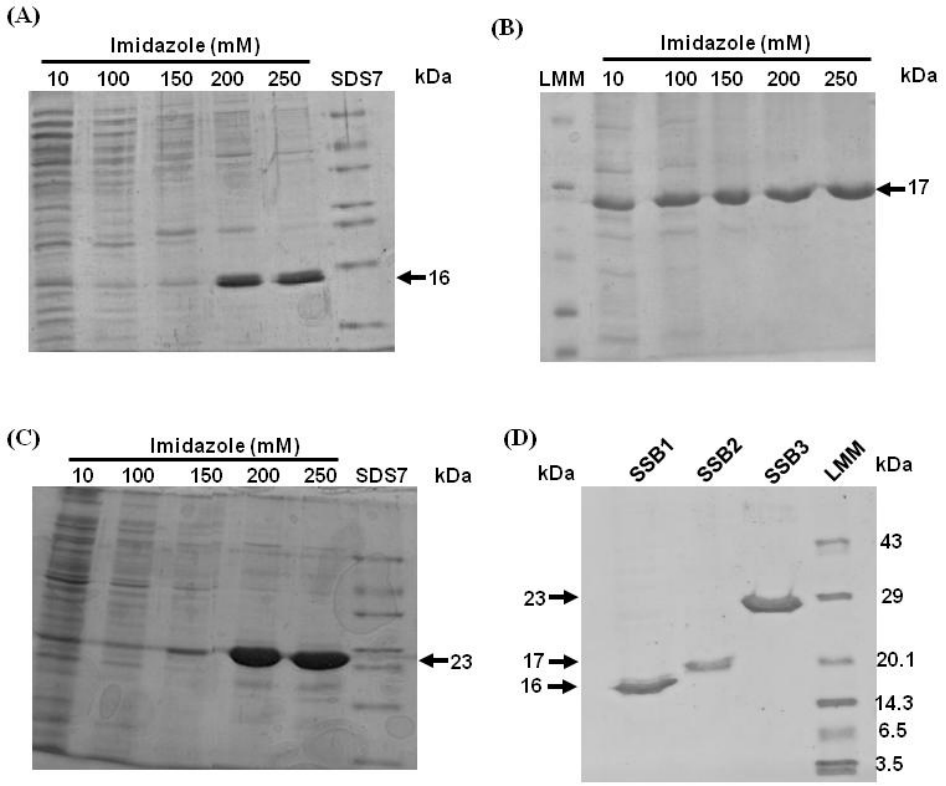

Fig. 3.8 Purification of *Anabaena* SSB proteins.

(A-C) The native protein extracts from *E. coli* cells overexpressing the three SSB proteins (A) SSB1, (B) SSB2 and (C) SSB3 were purified by Ni-NTA affinity chromatography using an imidazole gradient of 10 – 250 mM as indicated. (D) Pure SSB protein finally eluted with 1 M imidazole. The protein markers used was either SDS-7 or LMM (Low molecular weight Marker, B'lore Genie). The molecular masses of proteins in LMM are indicated in (D). The purified SSB proteins are indicated by arrows. Other details are as described in legend to Fig. 3.7.

in 20 mM Tris-HCl, pH 8.0, 500 mM NaCl buffer using an imidazole gradient (0-250 mM) **(Fig. 3.8 A-C)**. The proteins were finally eluted with 1 M imidazole. This fraction was dialysed against 20 mM Tris-HCl, pH 8.0, and 500 mM NaCl buffer to remove imidazole. The dialysed protein **(Fig. 3.8D)** was used for all subsequent biochemical assays. The purified (SSB1, SSB2 and SSB3) proteins migrated at 16, 17 and 23 kDa respectively **(Figs. 3.7 and 3.8)**, which is about 3 kDa more than the theoretical molecular mass due to the additional His_{10}- residues at the N-terminus.

3.2.3 Biochemical characterisation of *Anabaena* SSB Proteins

3.2.3.1 Oligomeric status of SSB Proteins

Bioinformatic analysis of the three *Anabaena* SSB proteins showed the presence of dimeric and tetrameric interfaces **(Fig 3.1)**. This is suggestive of the possibility of the formation of multimers by *Anabaena* SSB proteins. The multimerisation of all three proteins was checked by gel filtration as well as by glutaraldehyde cross-linking.

SSB1 and SSB2 proteins were eluted in two distinct fractions upon separation by gel filtration chromatography using Superdex 200 HR **(Fig 3.9)**. On the basis of the elution profile of standard proteins separated on the same matrix, the molecular mass of the different fractions was predicted as 14.1 kDa and 25.7 kDa for SSB1 and 14.5 kDa and 26.3 kDa for SSB2 **(Fig. 3.9)**, indicating formation of dimers in case of both SSB1 and SSB2 proteins. Higher molecular forms of these proteins were not detected even at higher concentrations.

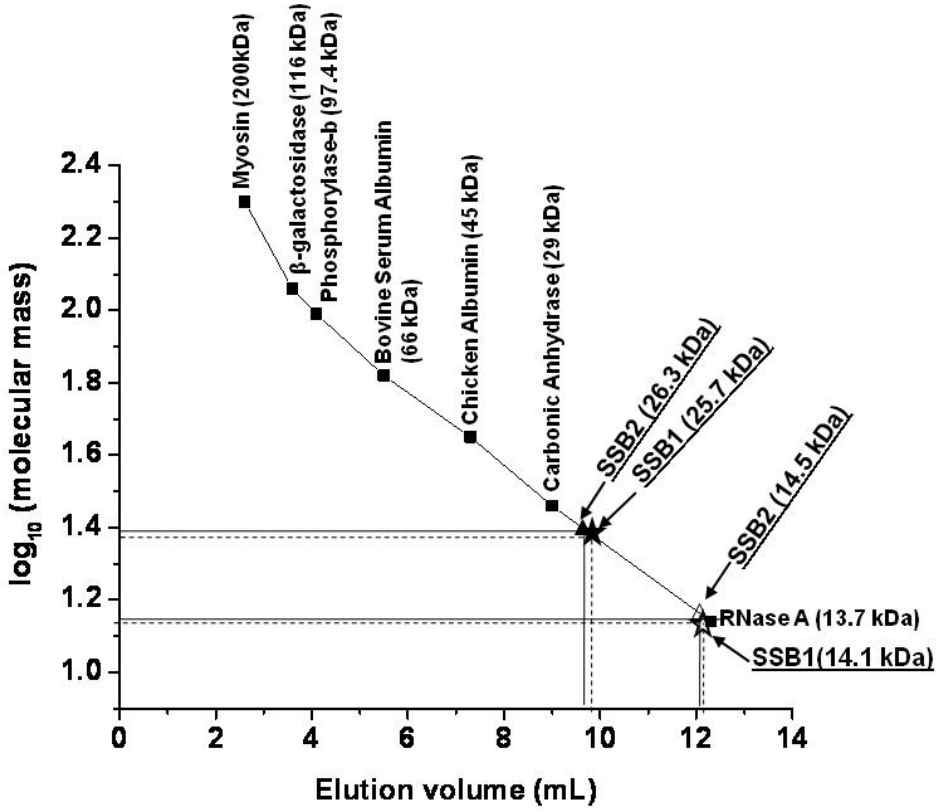

Fig. 3.9 Molecular mass determination of purified native *Anabaena* SSB1 and SSB2 proteins.

Elution profile of purified native SSB1 and SSB2 proteins in gel filtration chromatography using Superdex 200HR (10/30) matrix. A standard graph using the following standard proteins: [Myosin (200 kDa), β-galactosidase (116 kDa), Phosphorylase-b (97.4 kDa), Bovine Serum Albumin (66 kDa), Chicken Albumin (45 kDa), Carbonic Anhydrase (29 kDa) and RNaseA (13.7 kDa)] was drawn to calculate the molecular mass of the eluted native *Anabaena* proteins depending on their elution volume. The position of the eluted proteins has been depicted by 'star' and 'triangle' symbols. The vertical and horizontal lines from the two symbols indicate the elution volume and the corresponding log of molecular mass.

The SSB3 protein **(Fig. 3.10A)** eluted in two fractions, which on the basis of standard graph was calculated to be 23 kDa and 44 kDa **(Fig. 3.10B)** corresponding to monomeric and dimeric molecular mass. Since, His-tag is known to interfere with oligomerisation of proteins and quite often give rise to pseudo oligomers, attempts were made to cleave the His-tag from all the three SSB proteins. While this could not be achieved for SSB1 and SSB2 proteins, in case of SSB3 the His-tag could be removed after treatment with Factor Xa **(Fig. 3.10A)**. In the absence of His-tag, SSB3 existed predominantly as a 41 kDa dimer **(Fig. 3.10C)**. Irrespective of the absence or presence of His-tag, a tetrameric form could not be detected for SSB3 **(Fig. 3.10B and C)**.

Confirmation of the multimeric status of the three SSB proteins was further carried out by cross-linking the native proteins with glutaraldehyde and subsequent separation by SDS-PAGE. Upon cross-linking, in addition to the monomeric forms of the respective SSBs, the dimeric forms corresponding to 32 kDa for SSB1 **(Fig 3.11A)**, 34 kDa for SSB2 **(Fig 3.11B)** and 41 kDa for SSB3 **(Fig 3.11C)** were detected. The level of the dimeric form was lowest for SSB3. Hence, the cross-linking was carried out in the presence of M13 ssDNA to check if the dimeric and tetrameric forms were stable only in the presence of a ssDNA substrate. Addition of M13 ssDNA resulted in an increase in the levels of the dimeric 41 kDa form and a faint band corresponding to ~82 kDa or a tetrameric form could also be detected **(Fig 3.11C)**. This suggested that SSB3 may attain the native multimeric conformation preferably in the presence of ssDNA.

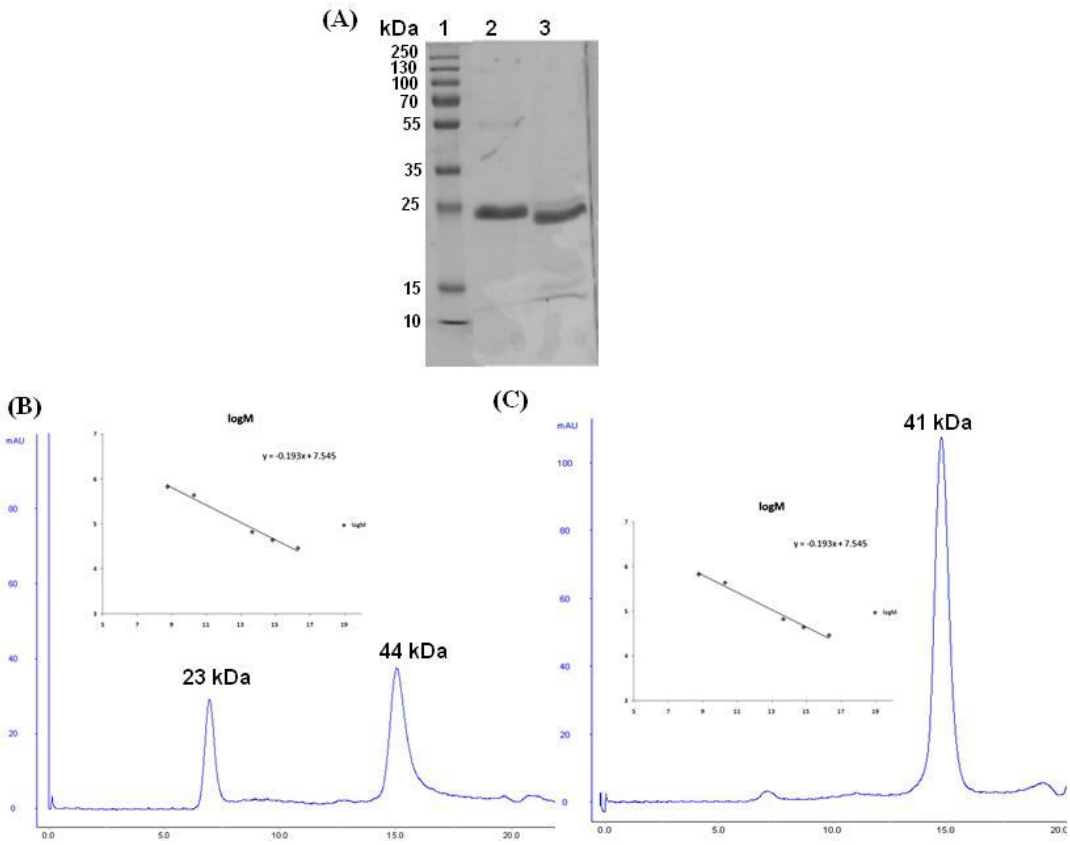

Fig. 3.10 Molecular mass determination of purified native *Anabaena* SSB3 proteins with or without His-tag

(A) Native purified SSB3 protein with His-tag (lane 2) and without His-tag (lane 3). His-tag was removed using Factor Xa. Lane 1 is protein molecular weight marker. Other details are as described in legend to Fig. 3.7. (B and C) Elution profile of purified native (B) SSB3 protein with His-Tag, and (C) SSB3 protein without His-tag, subjected to Gel filtration chromatography using Superdex 200HR matrix. The peaks corresponding to monomer and dimer are indicated along with the calculated molecular mass. The standard graph used for calculation of the molecular mass is shown in the inset.

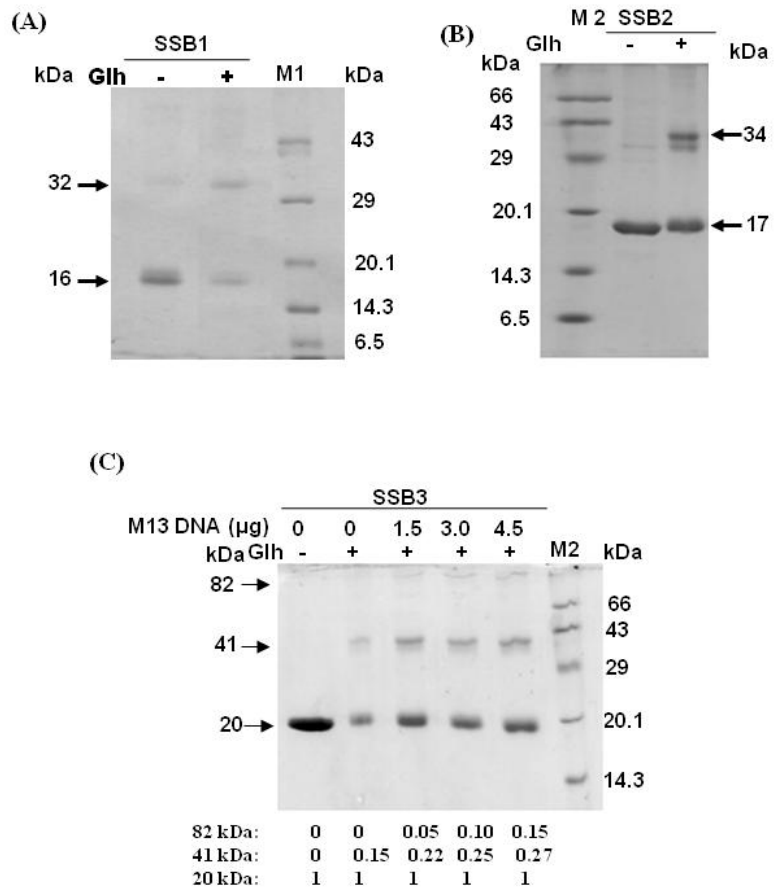

Fig. 3.11 Glutaraldehyde (Glh)-aided cross-linking of native purified *Anabaena* SSB proteins.

The purified native *Anabaena* proteins (A) SSB1, (B) SSB2, and (C) SSB3 were cross-linked with glutaraldehyde in the presence or absence of M13 ssDNA as indicated. The proteins were electrophoretically separated on 12% SDS-polyacrylamide gel followed by staining with Coomassie Brilliant Blue (CBB) G-250. The molecular mass of the protein markers used (M1 and M2) are written to the immediate (right/left) of the marker lane. Different molecular forms of the native *Anabaena* proteins are indicated by the arrows. The levels of 41 and 82 kDa SSB3 protein relative to the 20 kDa SSB3, taken as 1 is shown below Fig. 'C'.

3.2.3.2 DNA-binding properties of *Anabaena* SSB proteins

The ability of *Anabaena* SSB proteins to bind ssDNA was assessed using two techniques, namely fluorescence quenching and Electrophoretic Mobility Shift Assay (EMSA). Fluorescence quenching refers to decrease in the intrinsic fluorescence of a protein upon interaction with another molecule, such as ssDNA in case of SSB proteins. Intrinsic fluorescence of a protein arises from the presence of tryptophan, tyrosine and phenylalanine on the surface of the protein.

All three *Anabaena* SSB proteins exhibited maximum excitation at 280 nm and showed emission maxima at 310 nm for SSB1, 340 nm for SSB2 and 335 nm for SSB3 **(Fig. 3.12A)**. Upon addition of ssDNA in addition to different extent of quenching of fluorescence, a shift in wavelength of 3 nm to the lower side for SSB1 and 10 nm to the higher side for SSB2 was also observed **(Fig. 3.12A)** indicating interaction. A homopolymer poly(dT) was used as the ssDNA substrate since it does not form secondary structures. The intrinsic fluorescence of *Anabaena* SSB protein was found to decrease upon addition of poly(dT) till a point of saturation leading to a plateau, where no further change in fluorescence was obtained.

The change in fluorescence was plotted either as (i) percent relative fluorescence [$(F_e/F_i \times 100)$, where F_e is the fluorescence observed after addition of poly (dT) and F_i is the initial fluorescence of the protein] Vs concentration of poly (dT) used **(Fig. 3.12)** or (ii) Quenching [$\Delta F/F_i$, $\Delta F = F_i - F_e$] Vs the ratio of concentration of poly (dT) and SSB used **(Fig. 3.14)**. The relative fluorescence Vs Poly (dT) concentration graph **(Fig. 3.12)** was used to estimate the binding constant, which was calculated as the inverse of the concentration of poly (dT) at which 50% quenching of fluorescence was observed.

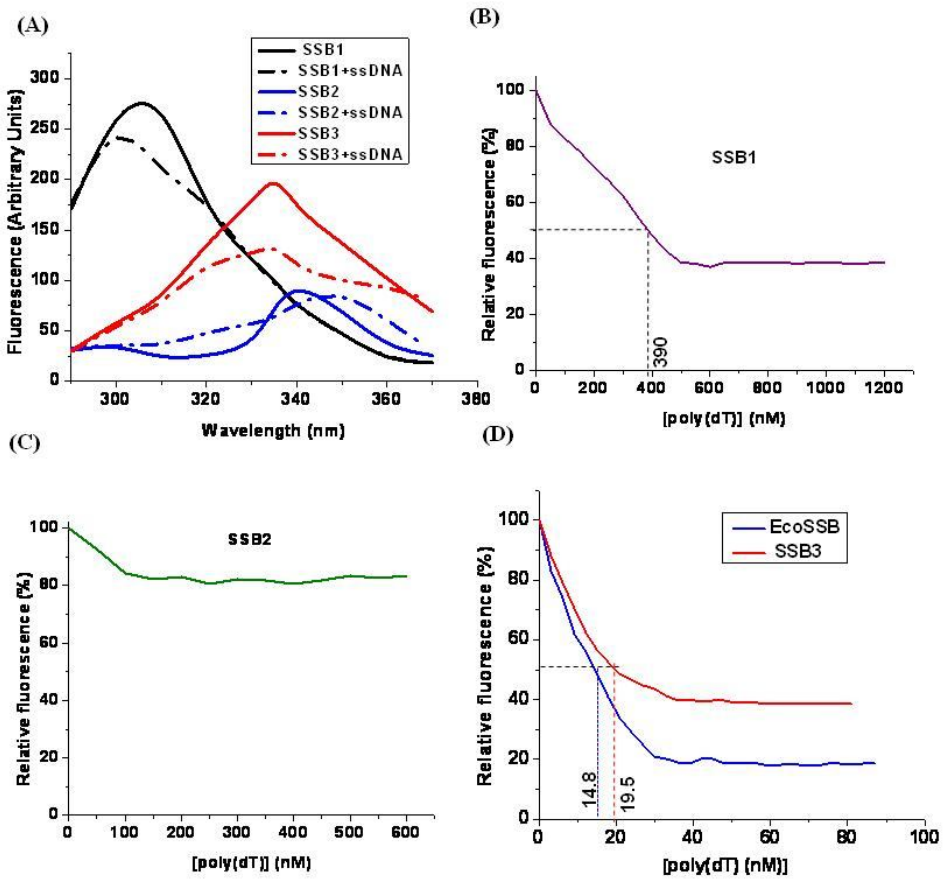

Fig. 3.12 Relative quenching of fluorescence of native purified *Anabaena* SSB proteins and EcoSSB as a function of ssDNA concentration.

(A) Change in fluorescence spectrum of SSB proteins upon addition of ssDNA. (B-D) Quenching of fluorescence in 20 mM NaCl as a function of poly(dT) concentration of (A) SSB1, (B) SSB2, and (C) SSB3 and purified EcoSSB (commercially available, Sigma) proteins represented as relative fluorescence, considering the observed fluorescence in the absence of any poly(dT) as 100%. The horizontal line designates the point on the graph wherein relative fluorescence is 50% and the corresponding vertical line indicates the concentration of poly(dT) at which it is achieved. Reciprocal of this concentration corresponds to the binding constant of the protein for poly(dT).

The relative fluorescence of SSB1 protein decreased to a maximum of 40% with ~450 nM poly(dT) **(Fig. 3.12B)**, that of SSB2 protein to a maximum of 80%, even at high concentrations of 600 nM of poly (dT) **(Fig 3.12C)**, while fluorescence of SSB3 protein decreased to about 40% at much lower concentrations (~35 nM) of poly(dT) **(Fig. 3.12D)**. The fluorescence of EcoSSB decreased to about 20% under the same experimental conditions **(Fig. 3.12D)**. The concentration of poly(dT) at which 50% quenching was observed was found to be 390 nM, 19.5 nM and 14.8 nM respectively for SSB1, SSB3 and EcoSSB **(Figs. 3.12B and D)**. The binding constant was thus calculated as $2.56 \pm 0.4 \times 10^6\ M^{-1}$ for SSB1, $5.13 \pm 0.71 \times 10^7\ M^{-1}$ for SSB3 and $6.76 \times 10^7\ M^{-1}$ for EcoSSB. The experimental value for EcoSSB was comparable to that reported in literature ($5.5 \pm 1.5 \times 10^7\ M^{-1}$) (185). The binding constant has been calculated as an average of three independent experiments, though the graphs in **Fig. 3.12** are representative of one such experiment.

The inability of poly(dT) to quench the fluorescence of SSB2 suggested weak interaction between the two. This could be either because SSB2 recognise secondary structures or it does not bind ssDNA in spite of having an OB-fold. To test this M13 ssDNA was used as a substrate and the quenching of fluorescence of all three *Anabaena* SSB proteins analysed at both low (20 mM) and high (100 mM) NaCl concentration. Most efficient quenching of fluorescence by M13 ssDNA was achieved for SSB2 **(Fig. 3.13)**. The quenching of fluorescence was higher at 100 mM NaCl compared to 20 mM NaCl and found to be 50% and 20% respectively for SSB1, 80% and 60% respectively for SSB2 and 20% and 0% respectively for SSB3 **(Fig. 3.13)**.

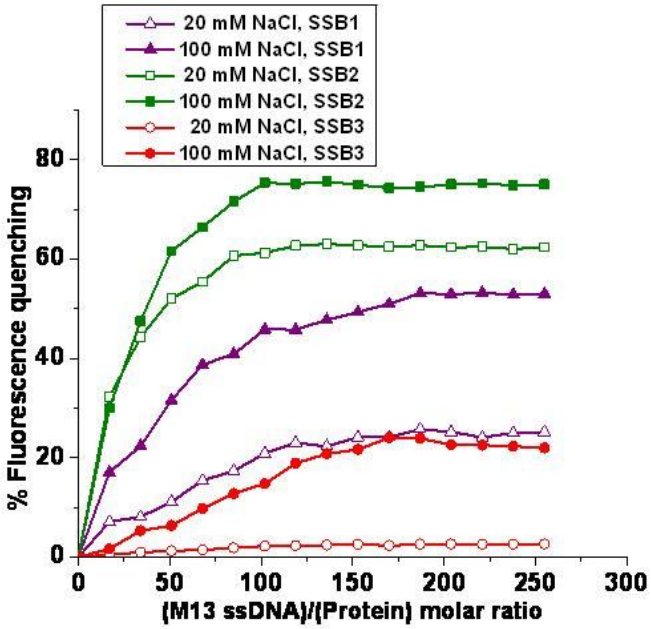

Fig. 3.13 Relative quenching of fluorescence of native purified *Anabaena* SSB proteins as a function of M13 ssDNA concentration.

Percent fluorescence quenching of SSB1, SSB2 and SSB3 proteins as a function of molar ratio of M13ssDNA and protein in the presence of 20 mM or 100 mM NaCl. The fluorescence quenching in the absence of M13ssDNA is considered as 0%.

The second graph **(Fig. 3.14)** was used to calculate the occlusion size, which is the value of the ratio of concentration of poly(dT) and SSB at which saturation of fluorescence quenching was observed. Irrespective of the NaCl concentration, saturation of quenching of fluorescence of SSB1 was observed when the ratio of concentration of poly(dT) to that of SSB1 was about 54-55 **(Fig. 3.14A)**, indicating an occlusion size of 54-55 nts for SSB1. Since no significant quenching of fluorescence of SSB2 with poly(dT) was observed **(Fig. 3.14B)**, its occlusion site could not be calculated. Quenching of fluorescence of both SSB3 and EcoSSB with poly(dT) showed distinct pattern based on NaCl concentration **(Figs. 3.14C and D)**. Based on this, the occlusion site was calculated as 35.5 and 65.9 at 20 mM and 100 mM NaCl respectively for SSB3 **(Fig. 3.14C)** and 36 and 72 for EcoSSB **(Fig. 3.14D)** at 20 mM and 100 mM NaCl respectively.

The binding of *Anabaena* SSB proteins was further confirmed by carrying out EMSA studies using a 75-mer ssDNA as the substrate. Maximum retardation in the mobility was observed with SSB3 and at much lower concentration than the other two SSB proteins **(Fig. 3.15)**. Decrease in the mobility of ssDNA was observed upon addition of 0.16 µg of SSB1 **(Fig. 3.15A)**. Retardation in the mobility of the 75-mer ssDNA with SSB2 was observed only at high concentrations of the protein (1.2 µg) **(Fig. 3.15B)** confirming weak interactions. The higher binding affinity of SSB3 for ssDNA was further confirmed by EMSA, requiring only 0.15µg SSB3 for complete retardation of the mobility of the ssDNA **(Fig. 3.15C)**.

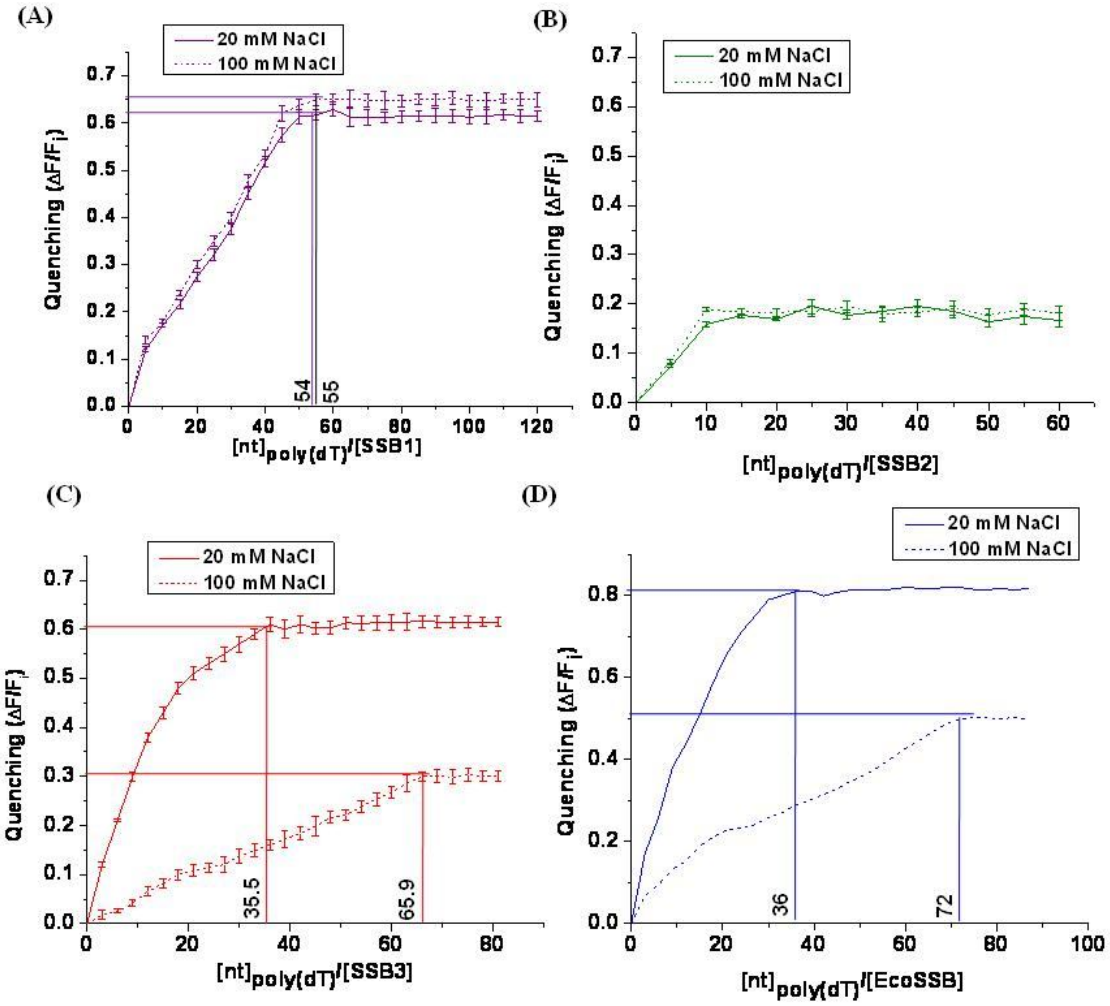

Fig. 3.14 Quenching of fluorescence of native purified *Anabaena* SSB proteins compared with that of EcoSSB.

Quenching of fluorescence of (A) SSB1, (B) SSB2, (C) SSB3 and (D) EcoSSB proteins in the presence of 20 mM or 100 mM NaCl expressed as a ratio of change in fluorescence (ΔF) and initial fluorescence (F_i), plotted as a function of ratio of concentrations of poly(dT) and protein. The horizontal lines indicate the point of saturation and the vertical lines drawn from the point of saturation indicate the probable length of ssDNA bound by one molecular unit of the protein.

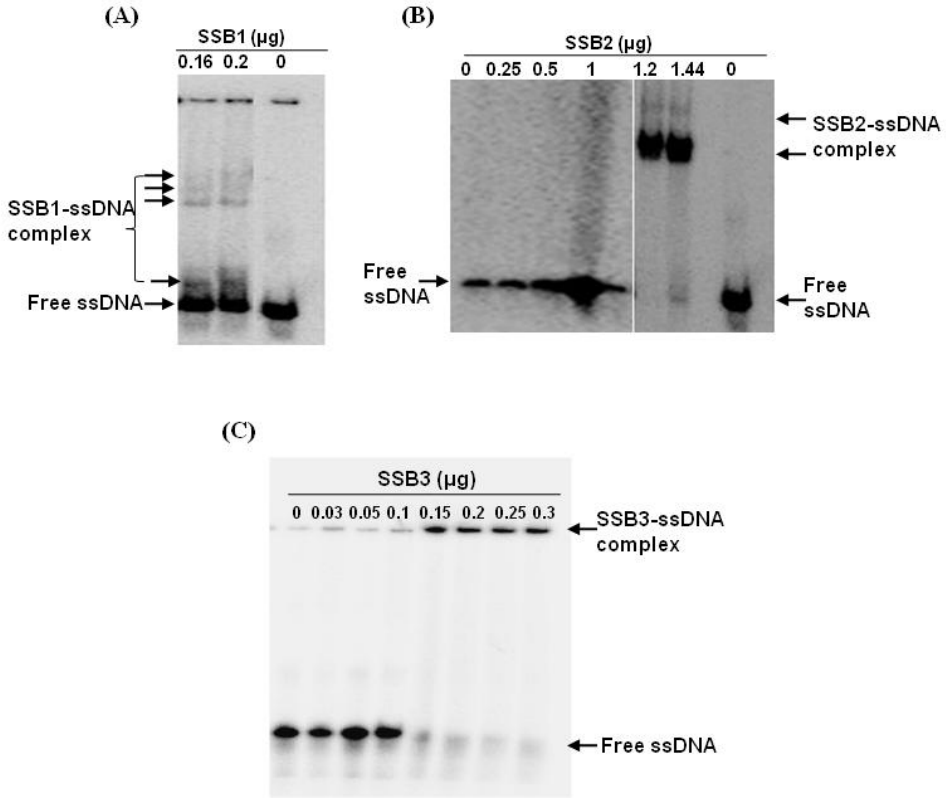

Fig. 3.15 Binding of *Anabaena* SSB proteins with 75-mer ssDNA.

Electrophoretic Mobility Shift Assay (EMSA) of a γ-^{32}P-ATP labelled 75-mer oligonucleotide in the presence of different concentrations of (A) SSB1, (B) SSB2 and (C) SSB3 proteins. Following *in solution* interaction, the assay mix was separated by 6% non-denaturing PAGE in 1X TBE and radioactive gel imaged using a phosphorimager. The free ssDNA substrate and the different ssDNA-protein complexes formed are indicated.

3.3 DISCUSSION

Bacterial SSB proteins which are about 170-180 amino acids long are characterised by the presence of an ~100 amino acid long OB-fold, followed by a P/G rich region which separates the positively charged OB-fold from the negatively charged acidic tail at the C-terminus (39). The only known exception to this is the ~117 amino acid long alternate SSB of *B. subtilis* and some of the naturally competent bacteria (105) and 141-142 amino acid long SSB of the thermophilic bacteria, *Thermotoga* sp. (185). *Anabaena* 7120 has three SSB-like proteins, of which SSB1 (Alr0088) and SSB2 (Alr7579) are naturally truncated SSBs with only the OB-fold and annotated as SSB in the genome database **(Fig. 3.1)**, while SSB3 (All4779) is full length and resembles typical bacterial SSB in having all three regions **(Fig. 3.1)**, but is annotated as a hypothetical protein. This was possibly due to the low and scattered identity of SSB3 at amino acid level with other bacterial SSBs. The P/G rich linker region of SSB3 differs from that of EcoSSB in being proline rich as against glycine rich in case of EcoSSB **(Fig. 3.3)**. While multiple glycine residues allow flexibility in structure, multiple proline residues provide rigidity and kinks in the structure and thus no ordered structure results in either gly-rich or pro-rich regions (187).

Dimeric and tetrameric interfaces were bioinformatically predicted for all the three SSBs of *Anabaena*, conforming to the known oligomerisation features in bacterial SSBs. However, HPLC-based gel filtration **(Figs. 3.9 and 3.10)** as well as glutaraldehyde based cross-linking experiments **(Fig. 3.11)** suggested formation of only dimeric forms for SSB1 and SSB2, while SSB3 could tetramerise to some extent in the presence of ssDNA **(Fig. 3.11)**. The only known functional dimeric SSB is from *D. radiodurans*.

However, the protomers of DrSSB is twice the size of *E. coli* SSB and contains two OB-folds and as a result a total of four OB-folds will be available for each SSB molecule of *D. radiodurans* (35). The presence of OB-fold signifies ssDNA/RNA binding ability (27); however, the availability of only one or two OB-folds per SSB molecule of *Anabaena* raised doubts on their ability to bind ssDNA efficiently. The ability of all three *Anabaena* SSB proteins to bind different forms of ssDNA [short oligos, poly(dT) and M13 ssDNA] was assessed by EMSA and fluorescence quenching techniques **(Figs. 3.12-3.15)**. Both these studies revealed that SSB3 has the highest affinity for binding to both short oligonucleotides as well as poly(dT), but not to M13 ssDNA, which attains secondary structure **(Figs. 3.12, 3.13 and 3.15)**.

In general, the absence of C-terminal acidic tail is known to result in increase in binding affinity of SSB to ssDNA (12, 186). However, the reverse was found to be true for *Anabaena* 7120, with SSB3 which has an acidic tail having 10-fold higher binding affinity than SSB1 which lacks the acidic tail. The possible reason could be the absence of P/G-rich region in SSB1 of *Anabaena*, which may also be contributing to the binding affinity. The earlier data (12, 186) is based on SSB proteins lacking only the C-terminal acidic tail. SSB2, on the other hand, exhibited very weak interactions with homo oligopolymers and short hetero-oligopolymers, but significantly higher interaction with long hetro-oligopolymers possessing secondary structure, such as M13 ssDNA **(Fig. 3.13)**. The quenching of fluorescence of SSB2 was not observed with thermally denatured M13 ssDNA. M13 ssDNA is known to form secondary structures (188), which are disrupted at high temperatures. This suggested that SSB2 may be recognizing secondary structures formed with long ssDNA rather than short stretches of ssDNA.

Since SSB2 neither binds short ssDNA which is part of loop nor dsDNA, which is part of the stem of a secondary structure, it is speculated that it may be recognising the overall secondary structure rather than the hairpin or loop individually.

SSB1 and SSB3 not only differed in their binding affinity, but also in their binding mode. SSB1 exhibited a single binding mode of 54-55 nucleotide **(Fig. 3.14A)**, which is the rarest of the three known binding modes of EcoSSB and designated as $[SSB]_{56}$ (39). The SSB3, on the other hand, exhibited two distinct binding modes **(Fig. 3.14C)**, similar to the two major binding modes of *E. coli* SSB, which interacts with either 35 nucleotides or 65 nucleotides (39), and also shown in **Fig. 3.14D**. Since, the $(SSB3)_{65}$ binding mode would require presence of SSB3 in a tetrameric form, which is not the predominant confirmation, the extent of binding by SSB3 in this mode is relatively slower than in the $(SSB3)_{35}$ mode wherein a dimeric form would suffice, in which two OB-folds are available. These results indicate that of the three *Anabaena* SSB proteins, SSB3 exhibits binding affinity and binding modes similar to that by *E. coli* SSB.

Thus, SSB3 is the major typical bacterial SSB of *Anabaena* 7120 in terms of structural domains and binding to ssDNA. The genes coding for the two atypical truncated annotated SSB proteins, SSB1 and SSB2 may have arisen due to gene duplication as suggested for PriB, a dimeric protein with only a single OB-fold per PriB molecule and capable of binding ssDNA (189), and may be involved in other functions such as replication and recombination. Another possibility is that SSB1 or/and SSB2 could perform function similar to SsbB of *B. subtilis*, which is involved in development of competence by protecting the incoming DNA (106, 107). The unicellular cyanobacterium, *Synechocystis* PCC6803 has been shown to be naturally transformable

with the possible involvement of competence proteins, ComA (Slr0197) (190), and ComF (Slr0388) (191). Orthologs of these genes are also found in *Anabaena* 7120, annotated as *all3087* and *alr2926* respectively (http://microbedb.genome.jp/cyanoabse/Anabaena), suggesting the possibility of *Anabaena* being also naturally transformable. Whether the truncated SSB proteins, SSB1 and SSB2 function as PriB or BsSsb needs to be proven experimentally. The assessment of the expression and physiological roles of the *Anabaena* SSB proteins is discussed in the subsequent chapters.

Chapter 4

Stress-induced regulation of *Anabaena* *SSB proteins and identification of the regulatory regions of the corresponding genes*

4.1 INTRODUCTION

Most bacteria possess a single gene coding for single stranded DNA binding (SSB) protein, which is generally located in the vicinity of other DNA repair genes. However, several cases of multiple SSBs or their paralogs have been reported, which are widely distributed in the genome. The bacterial *ssb* genes are classified into four groups on the basis of their gene organization and number of *ssb* paralogoues (105). Group I includes bacteria which possess a single *ssb* gene located as part of *rpsF-ssb-rpsR* operon, similar to that in *B. subtilis*, while the bacteria with same gene organization but having multiple *ssb* paralogoues such as that in *Helicobacter pylori, Campylobacter jejuni* etc are classified into Group II. Bacteria having *ssb* gene divergently located with respect to *uvrA* and regulated by LexA, as in *E. coli* are placed under Group III. Group IV includes the remaining bacteria where in the *ssb* gene organization does not fall into either of the above three groups. The placement in this group is irrespective of whether the bacterium has a single *ssb* gene or multiple *ssb* paralogs. The nitrogen-fixing cyanobacterium, *Anabaena* sp. PCC7120 is classified into Group IV on the basis of the above classification (105).

Three modes of regulation of SSB expression have been reported in bacteria. The most common is the negative regulation of *ssb* gene by LexA, exemplified in *E. coli* wherein a two-fold induction of SSB is reported upon mitomycin C treatment (118). In *E. coli*, post-transcriptional regulation of SSB has also been reported wherein SSB binds to its own mRNA, thereby inhibiting further translation of the protein (120). In *D. radiodurans*, a conserved palindromic radiation desiccation response motif (RDRM)

acting as a *cis* element is involved in the transcriptional up regulation of the *ssb* genes (119).

4.2 RESULTS

4.2.1 Organisation of *ssb* genes in *Anabaena*

The three *ssb* genes of *Anabaena* 7120 are located at distinct positions on the chromosome. The *ssb1* gene annotated as *alr0088* is present at position 91483-91842 on the chromosome, *ssb2* (*alr7579*) at 74007-74390 and *ssb3* (*all4779*) at position 5694331-5694879. The genes flanking *ssb1* i.e. *all0087* and *all0089* are transcribed in a direction opposite to *ssb1*, which code for proteins involved in determination of cell shape **(Fig. 4.1)**. The gene organization indicates that *ssb1* is a monocistronic gene. The gene to the left of *ssb2* (*alr7579*) i.e. *all7578* is transcribed in the opposite direction with respect to *ssb2*, while three consecutive genes to its right, i.e. *alr7580, alr7581* and *alr7582* are transcribed in the same direction as *ssb2* **(Fig. 4.2)**. Both the genes flanking *ssb2* code for 'unknown proteins', whose nearest ortholog has been identified as an aminopeptidase and oxidoreductase respectively, while the other two genes (*alr7581* and *alr7582*), transcribed in the same direction as *ssb2* code for cell partitioning proteins **(Fig. 4.2)**. Being the first gene in a set of genes transcribed in the same direction, *ssb2* would be regulated by its own promoter. The immediate genes on either side of *ssb3* (*all4779*) i.e. *all4778* and *all4780* are transcribed in the same direction as *ssb3* **(Fig. 4.3)**, which may either be an independently transcribed gene or as part of a bicistronic or tricistronic operon. The genes flanking *ssb3* code for a probable monooxygenase (*all4780*) and ABC-transporter ATP binding protein (*all4778*) **(Fig. 4.3)**. Thus, in case of *Anabaena ssb*

genes, none of the flanking genes code for proteins involved in DNA repair, unlike that observed in other bacteria.

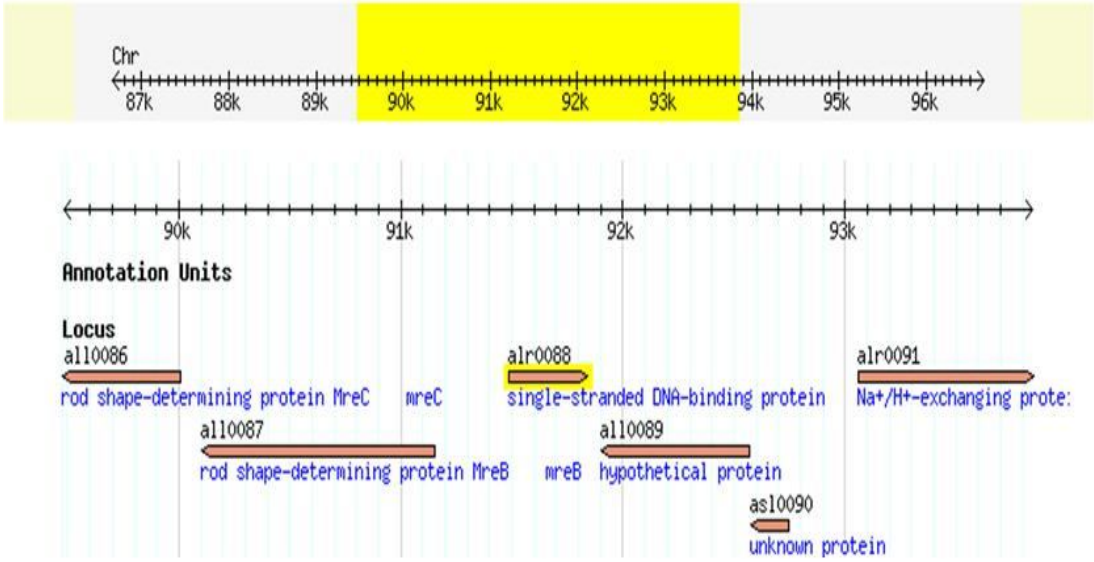

Fig. 4.1 Genomic organization of *Anabaena* 7120 *ssb1* (*alr0088*) gene.

The position and orientation of different genes in the vicinity of *alr0088* in a 5 kb region on the *Anabaena* genome. The position on the genome is represented by the scale in black. The first letter 'a' refers to *Anabaena* 7120, the second letter 'l' or 's' refer to gene size i.e. large or small (< 100 bases) respectively, and the third letter 'r' or 'l' refer to the direction of transcription i.e. right or left respectively. The number following the three letters is the gene number. The annotation of the different genes is given below each gene. The gene *alr0088* is highlighted in yellow.

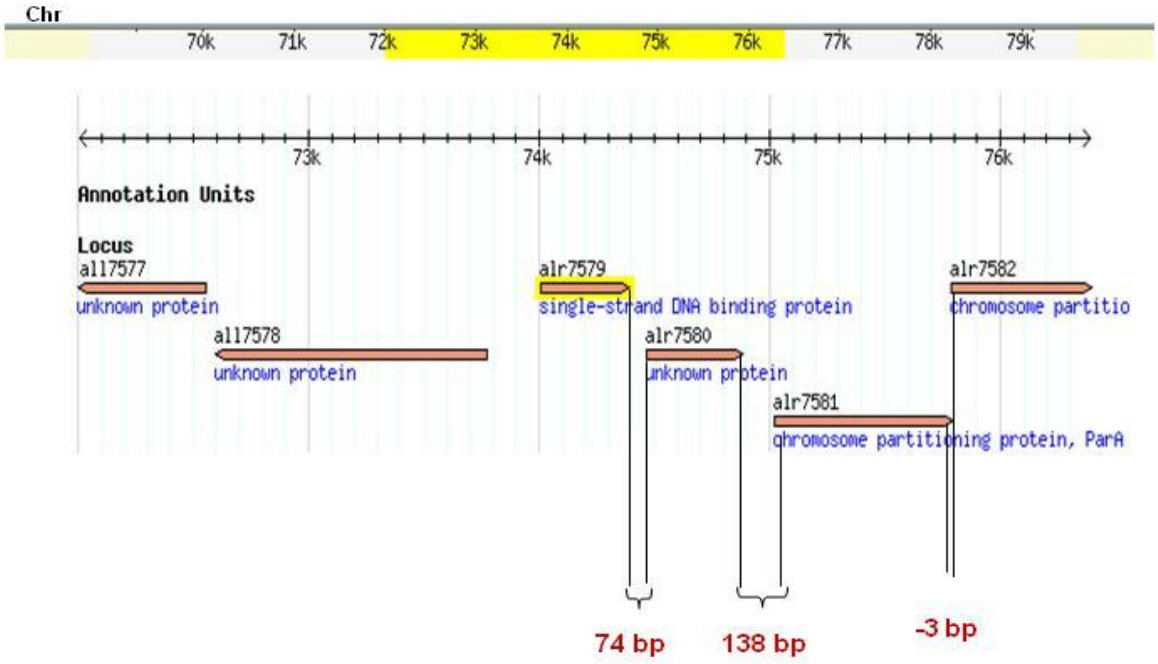

Fig. 4.2 Genomic organization of *Anabaena* 7120 *ssb2* (*alr7579*) gene.

The position and orientation of different genes in the vicinity of *alr7579* in a 5 kb region on the *Anabaena* genome. The gene *alr7579* is highlighted in yellow. The distance between the genes adjacent to *alr7579* and transcribed in the same direction is given in red. Other details are as described in legend to Fig. 4.1.

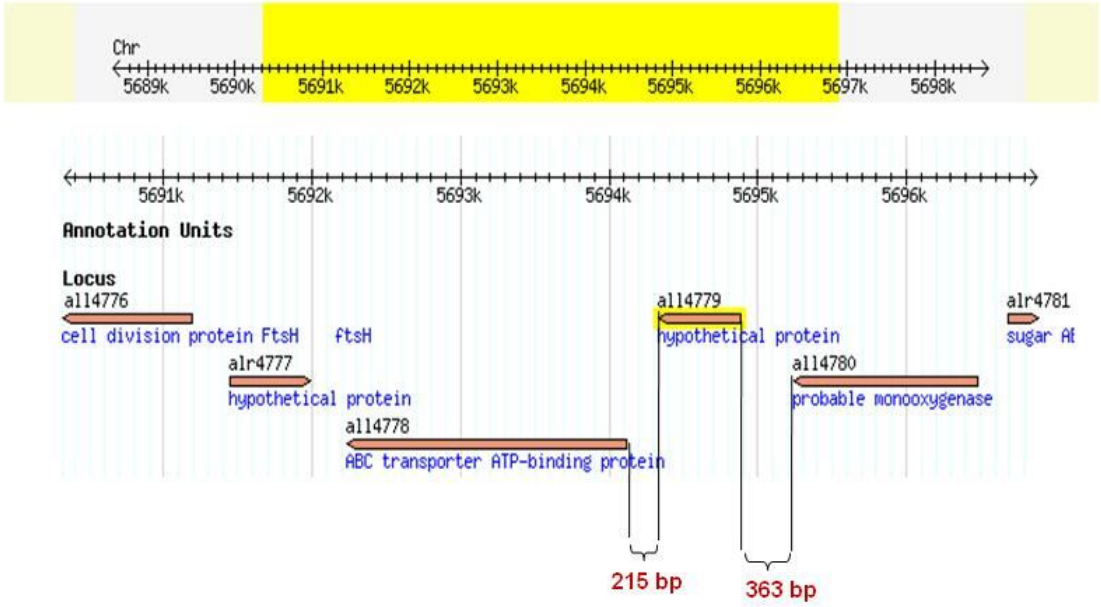

Fig. 4.3 Genomic organization of *Anabaena* 7120 *ssb3* (*all4779*) gene.

The position and orientation of different genes in the vicinity of *all4779* in a 5 kb region on the *Anabaena* genome. The gene *all4779* is highlighted in yellow. Other details are as described in legend to Fig. 4.2.

Northern blot analysis using *ssb* gene probes was carried out, but the transcript levels were possibly too low to be detected. Hence, a more sensitive method, Reverse Transcriptase (RT) PCR was carried out using *Anabaena* 7120 total RNA (0.5 μg) as a template and the respective *ssb* gene-specific primers. Single transcripts of sizes ~0.36 kb and ~0.38 kb were detected for *ssb1* and *ssb2* respectively **(lanes 1 and 2, Fig. 4.4A)** upon subjecting the total RNA to one step RT-PCR using (i) *ssb1*Fwd and *ssb1*Rev primers **(Table 2.3)** for *ssb1* transcript, and (ii) *ssb2*Fwd and *ssb2*Rev primers **(Table 2.3)** for *ssb2* transcript. These sizes corresponded to the gene size obtained upon PCR amplification of the genomic DNA using the same set of primers and Taq DNA Polymerase **(lanes 4 and 5, Fig. 4.4A)**. No amplicons were obtained upon subjecting total RNA to Taq DNA polymerase based PCR reaction using the same set of primers for each of the *ssb* genes **(lanes 6 and 7, Fig. 4.4A)**, confirming that there was no DNA contamination in the RNA preparation. One step RT-PCR of the RNA with *ssb3*Fwd and *ssb3*Rev primers **(Table 2.3)** resulted in a 0.55 kb transcript **(lane 4, Fig. 4.4B)**, which correspond to the gene size obtained using genomic DNA as template, same set of primers and Taq DNA Polymerase **(lane 2, Fig. 4.4B)**. Thus, transcription of all three *ssb* genes of *Anabaena* 7120 could be detected under normal growth conditions.

To analyse if *ssb3* (*all4779*) is part of a multicistronic operon, total RNA from *Anabaena* 7120 was subjected to RT-PCR using three different set of primers **(Table 2.3, Fig. 4.5A)** (i) 78 R (250 bp from the start codon of *all4778* oriented towards the start of gene) and 79 F (*ssb3*Fwd), (ii) 80 F (250 bp from the stop codon of *all4780* oriented towards stop codon of gene) and 79 R (*ssb3*Rev) and (iii) 78 R and 80 F. Genomic DNA was used as a positive control to test the three sets of combinations.

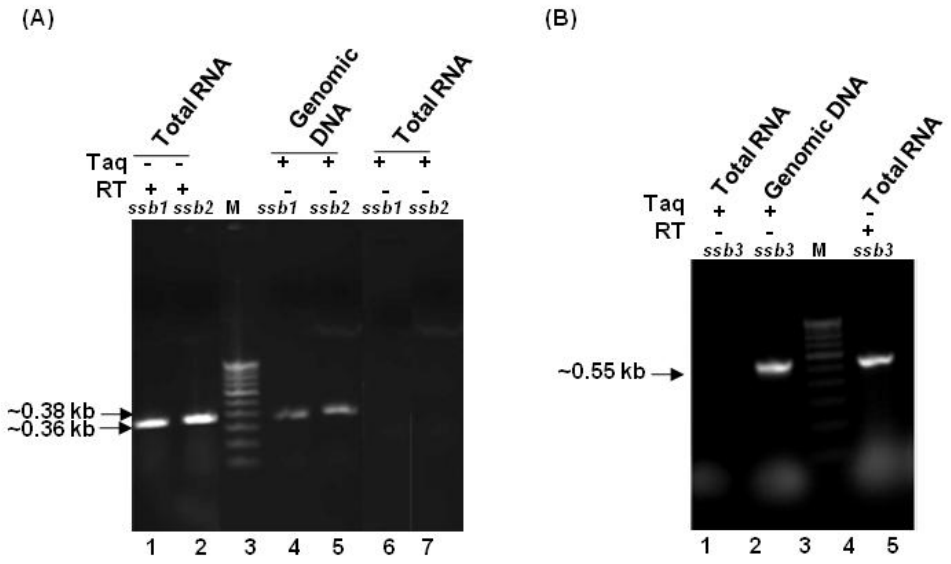

Fig. 4.4 Detection of transcripts corresponding to *Anabaena* 7120 *ssb* genes.

Total RNA was isolated from *Anabaena* 7120 cells grown under control and nitrogen-fixing conditions. Purified RNA (200 ng) was subjected to either one step reverse-transcriptase (RT) PCR or Taq DNA Polymerase (Taq)-based PCR using gene specific primers for (A) *ssb1* and *ssb2* and (B) *ssb3*. The positive control used was genomic DNA PCR amplified with Taq DNA Polymerase using the same set of primers and the negative control was PCR amplification of total RNA with Taq DNA Polymerase. The amplified fragments are indicated by arrows along with the approximate molecular mass. Lane M corresponds to 100 bp DNA ladder. Other details are as described in legend to Fig. 3.5.

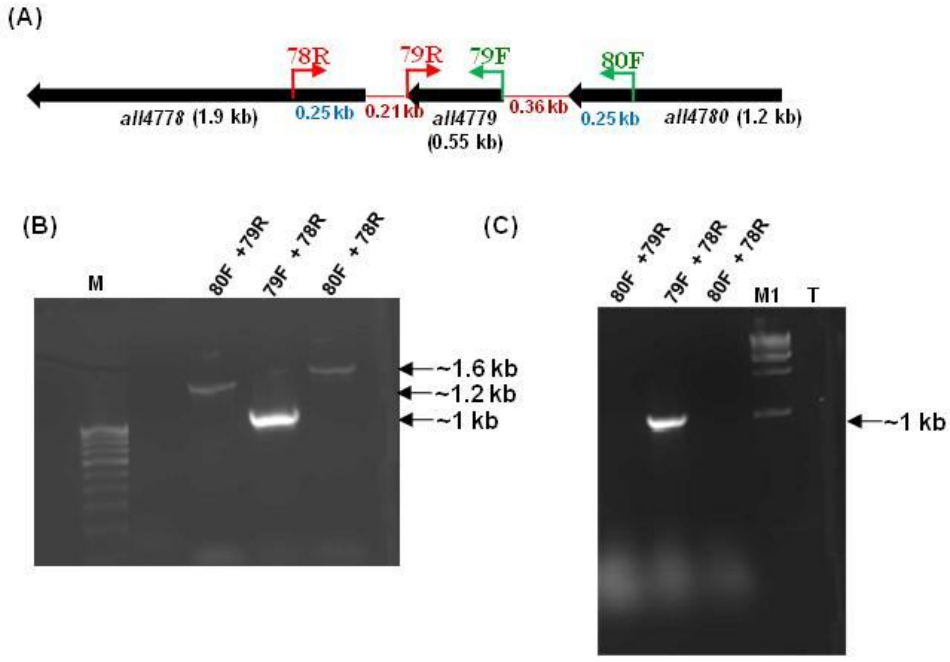

Fig. 4.5 Operon status of *all4779*.

(A) Schematic representation of *all4779* and its immediate neighbours. The length of the gene as well as intergenic regions is indicated. The positions of different primers used [*all4778*Rev (78R), *all4779*Rev (79R), *all4779*Fwd (79F) and *all4778*Fwd (78F)] is indicated. (B and C) PCR amplification with three sets of primers as indicated, using (B) Genomic DNA as template and Taq DNA Polymerase, and (C) Total RNA as the template and Reverse Transcriptase, except in lane T, wherein Taq DNA Polymerase was used. Lane M and M1 are 100 bp and 1 kb DNA ladders, respectively. Other details are as described in legends to Fig. 3.5 and 4.4.

The different amplicons obtained with genomic DNA were 1 kb, 1.2 kb and 1.6 kb respectively using the primer sets (i) 79F and 78R, (ii) 80F and 79R, and (iii) 80F and 78R **(Fig. 4.5B)**, which corresponds to the expected size. However, when RT-PCR was carried out with total RNA, a transcript of 1 kb was obtained only with 79F and 78R primers, while no transcripts were obtained with the other two primer sets **(Fig. 4.5C)**. This indicated that *all4778* and *all4779* are transcribed as a bicistronic operon from the *ssb3* gene promoter, while *all4780* is transcribed independently.

4.2.2 Prediction of putative promoters of *Anabaena ssb* genes

In silico analysis of the upstream regions of *ssb* genes was carried out using an online software program (http://molbiol-tools.ca/promoters.htm) indicating the presence of a promoter region with maximum probability for each of three *ssb* genes and the probable -10 and -35 as shown in **Fig. 4.6**. Since, most bacterial *ssb* genes are known to be regulated by LexA which binds to a palindromic LexA box near the promoter region, presence of such a box in the probable promoter regions of the three *ssb* genes of *Anabaena* 7120 was analysed. LexA box-like sequences were detected (indicated in green) in the promoter regions of both *ssb1* and *ssb2*, but not of *ssb3* **(Fig. 4.6)**. The LexA box of *ssb1* (**AGTAC**TTAT**GTACT)** was present immediately downstream to the putative -10 region **(Fig. 4.6A)**, while in case of *ssb2* **(ACT**CATA**AGT)**, it was present about 60-65 bases upstream of the putative -35 region **(Fig. 4.6B)**. The promoter region of *ssb3* was found to be AT-rich and the region 150-350 bp upstream of the start codon had AT content of 72% **(Fig. 4.6C)**.

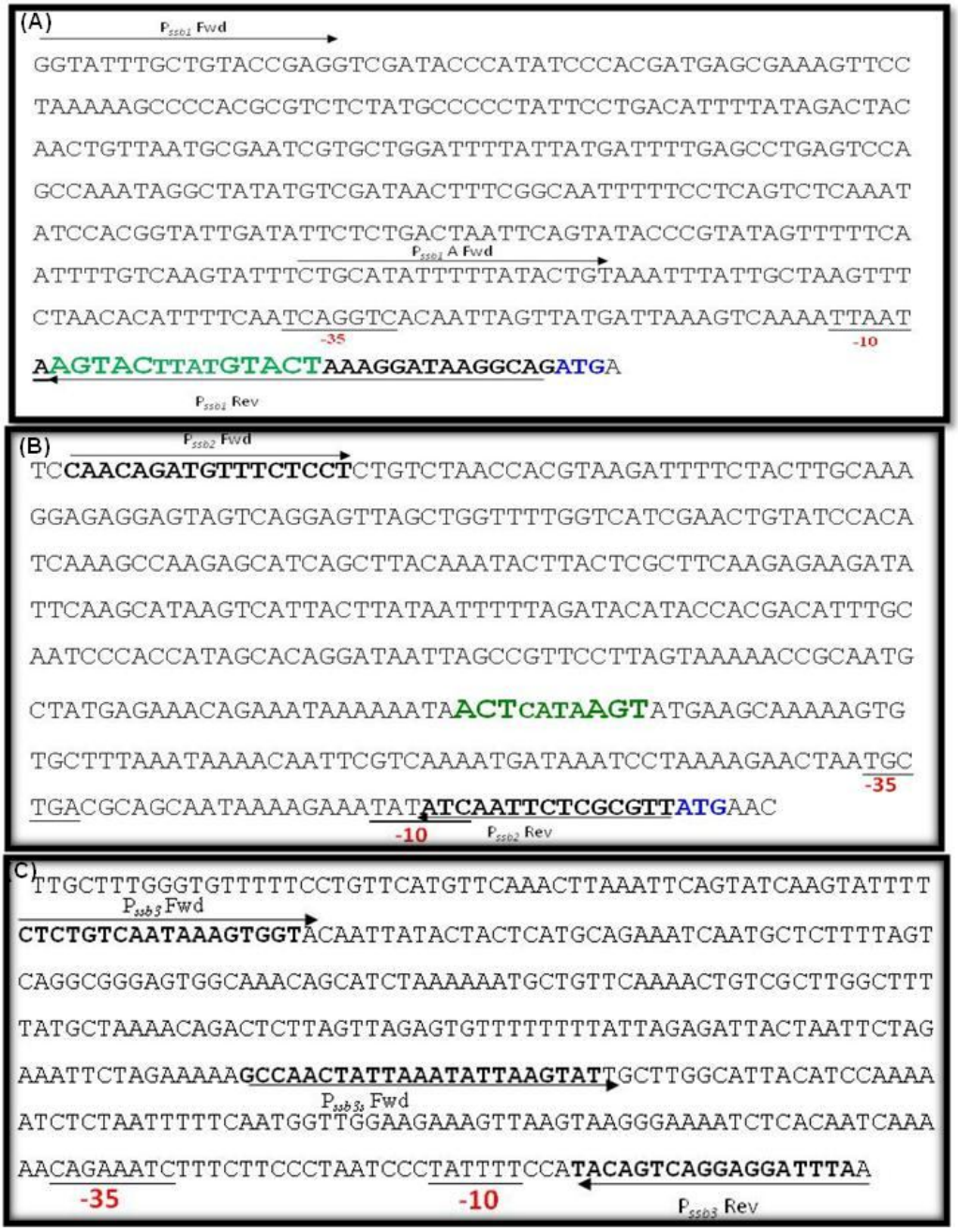

Fig. 4.6 DNA sequence of the putative promoters of *Anabaena ssb* genes

DNA sequence of regions upstream of (A) *ssb1*, (B) *ssb2* and (C) *ssb3* genes. The translational start site is indicated in blue; the putative -10 and -35 regions are underlined and indicated in red. The LexA box in (A) and (B) is indicated in green. The positions of different primers used are indicated.

A 400 bp region upstream of the translational start codon of ORF of *ssb1* and *ssb2* designated as P_{ssb1} and P_{ssb2} respectively and a 350 bp and 150 bp region upstream of translational start site of ORF of *ssb3* designated as P_{ssb3} and P_{ssb3s} respectively were individually amplified **(Fig. 4.7)** using $P_{ssb(1/2/3/3s)}$Fwd and $P_{ssb(1/2/3/3s)}$Rev primers **(Table 2.3, Fig. 4.6)**.

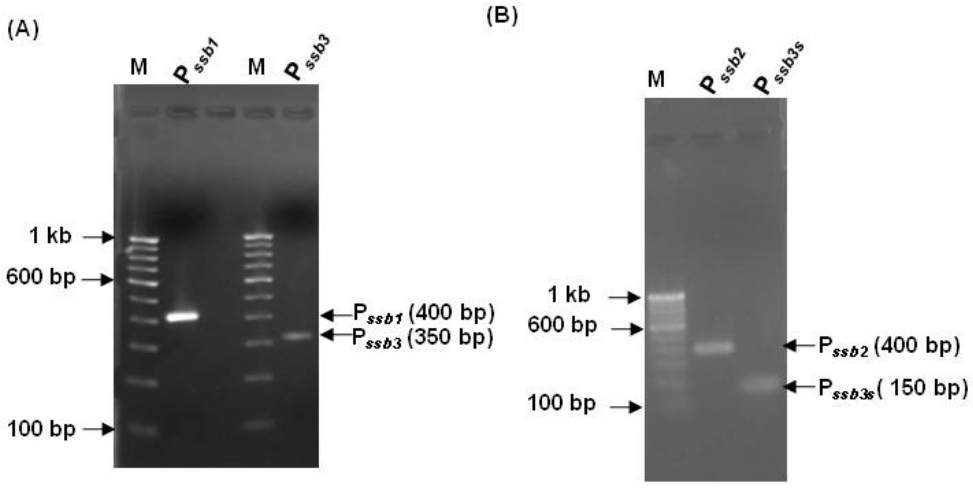

Fig. 4.7 PCR amplification of the putative promoters of *Anabaena ssb* genes

The putative promoter regions of (A) *ssb1* and *ssb3* and (B) *ssb2* and *ssb3* were PCR amplified from *Anabaena* 7120 genomic DNA using primers shown in Fig. 4.6. The primer sets used were (i) P_{ssb1} Fwd and P_{ssb1}Rev for P_{ssb1}, (ii) P_{ssb2} Fwd and P_{ssb2}Rev for P_{ssb2}, (iii) P_{ssb3} (-400) Fwd and P_{ssb3}Rev for P_{ssb3}, and (iv) P_{ssb1} (-154) Fwd and P_{ssb1}Rev for P_{ssb3s}. The different amplicons along with their sizes are indicated. Other details are as described in legend to Fig. 3.5.

The PCR fragments were individually digested with *Kpn*I and *Sac*I enzymes and ligated to the 11 kb *Kpn*I/*Sac*I digested pAM1956, a promoterless vector having *gfpmut*2 as a reporter gene which codes for Green Fluorescence Protein (GFP), as shown in **Fig. 4.8A**. Cloning of a functional promoter would be indicated by expression of green fluorescence by the recombinant bacterial cells. The ligation mix was transformed into *E.* coli DH5α cells and transformants were selected on LB Kan_{50} plates. The presence of inserts in the recombinant plasmids was confirmed by restriction digestion. A 0.4 kb insert corresponding to the *ssb1* and *ssb2* promoters and a 11 kb fragment corresponding to the vector were obtained upon digestion of the plasmid P_{ssb1}::*gfpmutII* and P_{ssb2}::*gfpmutII* respectively with *Kpn*I and *Sac*I **(Fig. 4.8B)**. Digestion of plasmids P_{ssb3}::*gfpmutII* and P_{ssb3s}::*gfpmutII* with *Kpn*I and *Sac*I resulted in the release of 0.35 kb and 0.15 kb inserts respectively, along with the 11 kb vector **(Fig. 4.8C)**.

The plasmid constructs $P_{ssb(1/2/3/3s)}$::*gfp* were individually introduced into *Anabaena* by conjugation using *E.* coli HB101 cells and the helper plasmids pRL441 and pRL623, also maintained in HB101. The exconjugants were selected on BG-11, N^+ Neo_{25} plates and designated as An(pAM-P_{ssb1}::*gfp*), An(pAM-P_{ssb2}::*gfp*), An(pAM-P_{ssb3}::*gfp*), An(pAM-P_{ssb3s}::*gfp*) respectively. Upon visualisation under a fluorescence microscope (λ_{Ex}: 470 nm, λ_{Em}: 508 nm), the vector control cells AnpAM (harbouring the plasmid pAM1956) exhibited red fluorescence, while the other recombinant *Anabaena* strains harbouring the different promoter constructs exhibited orange or green colour **(Fig. 4.9A)**.

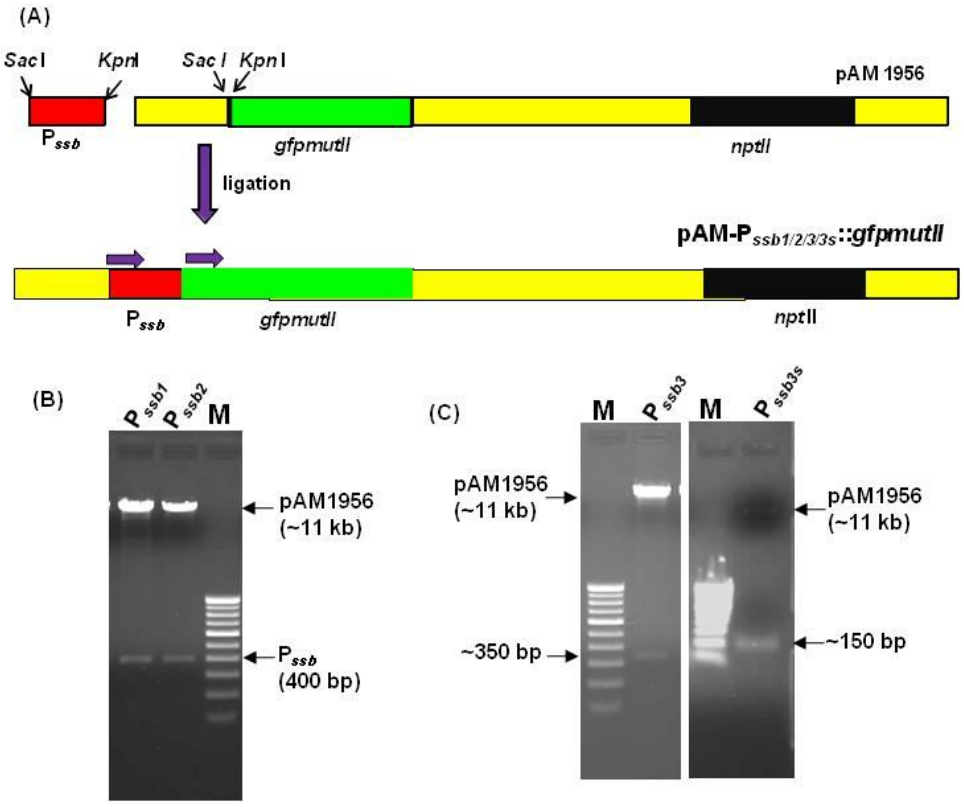

Fig. 4.8 Cloning of the putative promoters of *Anabaena ssb* genes into pAM1956

(A) Schematic representation of the cloning of the different promoter regions into the promoterless vector, pAM1956. The reporter gene, *gfpmutII* and the antibiotic marker gene, *nptII* are indicated as green and brown boxes in the plasmid pAM1956 shown as a yellow box. The amplified promoter region is shown as a red box. The restriction sites used for cloning are indicated. (B and C) Restriction digestion of the recombinant plasmids (B) P_{ssb1}*::gfpmutII* (P_{ssb1}) and P_{ssb2}*::gfpmutII* (P_{ssb2}), and (C) P_{ssb3}*::gfpmutII* (P_{ssb3}) and P_{ssb3s}*::gfpmutII* (P_{ssb3s}) with *Sac*I and *Kpn*I restriction endonucleases. The different fragments released along with their sizes are indicated. Lane M represents 100 bp DNA ladder. Other details are as described in legend to Fig. 3.5.

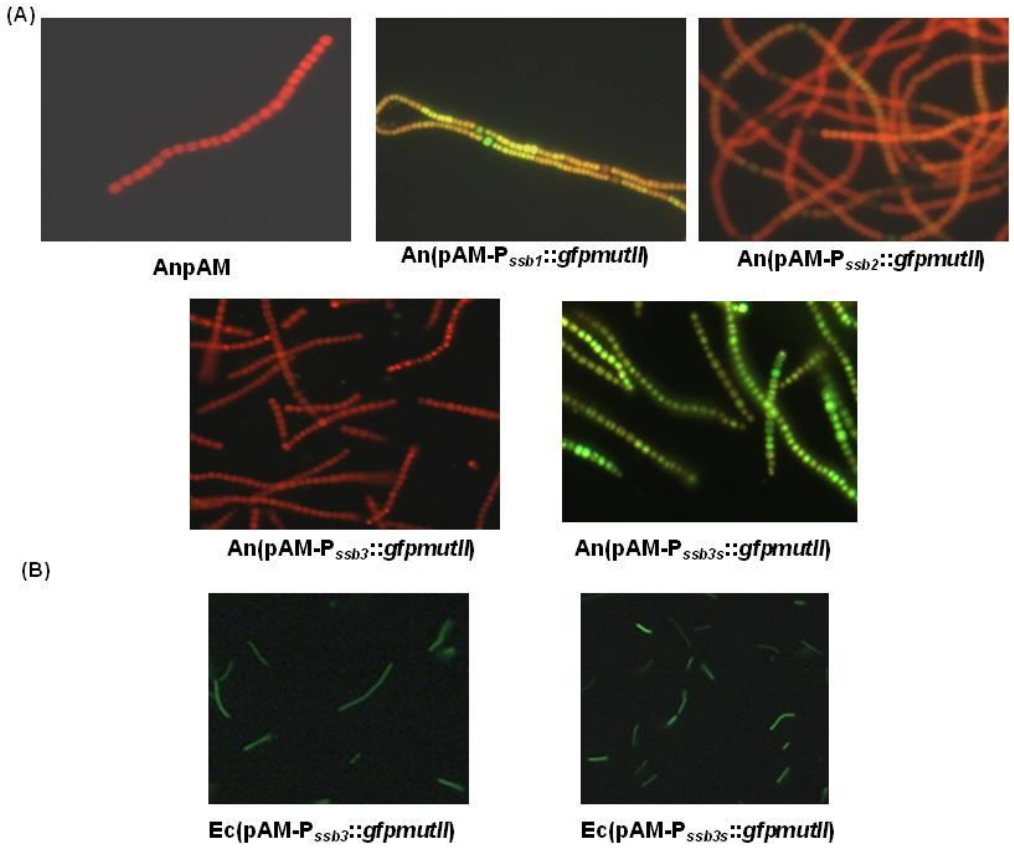

Fig. 4.9 Fluorescence micrographs of *Anabaena* and *E. coli* cells harbouring *ssb* promoter constructs.

(A) Fluorescence microphotograph (600 X magnification) of recombinant *Anabaena* strains harbouring either the empty vector, pAM1956 (AnpAM) or *ssb* promoter constructs namely pAM-P$_{ssb1}$*:: gfpmutII*, pAM-P$_{ssb2}$*::gfpmutII*, pAM-P$_{ssb3}$*::gfpmutII* and pAM-P$_{ssb3s}$*::gfpmutII*. (B) Fluorescence microphotograph (600 X magnification) of recombinant *E. coli* HB101 strains harbouring either pAM-P$_{ssb3}$*::gfpmutII and* pAM-P$_{ssb3s}$*::gfpmutII* plasmids. Fluorescence micrographs were obtained using Hg-Arc lamp (excitation 470 nm, emission 508 nm).

Based on the intensity of green fluorescence exhibited by the recombinant *Anabaena* strains, among the promoters having LexA box, the *ssb1* promoter exhibited stronger GFP expression than the *ssb2* promoter, while P_{ssb3s} was found to be the strongest promoter and P_{ssb3}, the weakest **(Fig. 4.9A)**. Unlike the significant difference in GFP fluorescence in *Anabaena* harbouring the two *ssb3* promoter constructs, P_{ssb3} and P_{ssb3s} **(Fig. 4.9A)**, in *E. coli* HB101 cells, GFP fluorescence was similar irrespective of the plasmid present i.e. $pAMP_{ssb3}$ or $pAMP_{ssb3s}$ **(Fig. 4.9B)**. The major difference in the two promoters is the absence of the AT-rich track located 150-350 bp upstream of the translation start codon of *ssb3* in P_{ssb3s}. Lack of inhibition of promoter activity by this region in *E. coli*, indicated that the AT-rich track may be regulated by a repressor protein specific to *Anabaena*.

A quantitative estimation of the promoter activity indicated that the basal activity for the vector control, AnpAM was about 20 units **(Fig. 4.10)**. GFP fluorescence increased to ~40, 120 and 180 units upon transcription from P_{ssb2}, P_{ssb1} and P_{ssb3s} promoters respectively, while no change in activity was observed with P_{ssb3} **(Fig. 4.10)**. The activity of all the promoters was monitored over a 10-day period under control growth and nitrogen-fixing conditions. The activity of P_{ssb1} and P_{ssb2} remained unchanged over a 4-day period and decreased thereafter **(Fig. 4.10**), indicating higher expression during logarithmic phase. On the other hand, activity of P_{ssb3s} continued to increase up to 7 days, reaching a maximum of 400 units and decreased thereafter during stationary phase **(Fig. 4.10)**.

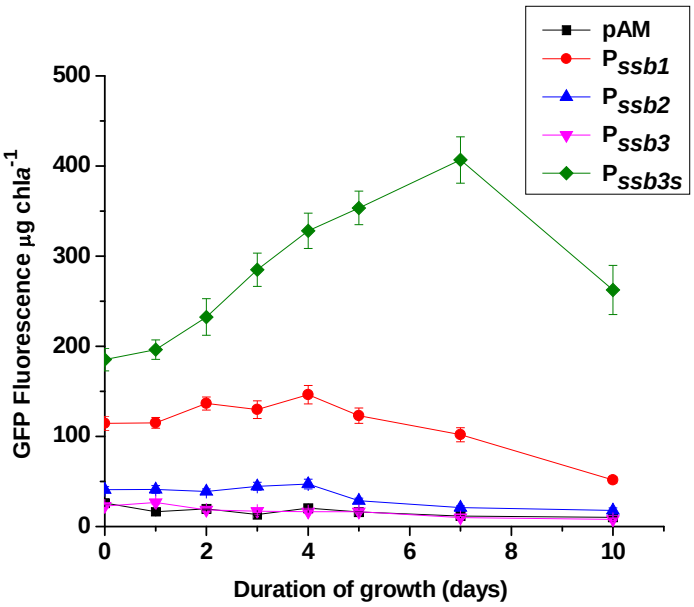

Fig. 4.10 Growth phase-dependent activity of *ssb* promoters in *Anabaena*

Fluorimetric analysis of GFP (Green Fluorescent Protein) expression levels in recombinant *Anabaena* cells freshly inoculated in BG-11, N^-, Neo_{25} growth medium and incubated for a period of 10 days under constant illumination and standing conditions. The different recombinant *Anabaena strains* used were AnpAM [pAM], An(pAM-P_{ssb1}*::gfpmutII*) [P_{ssb1}], *An(*pAM-P_{ssb2}*::gfpmutII*) [P_{ssb2}], An(pAM-P_{ssb3}*::gfpmutII*) [P_{ssb3}], and An(pAM-P_{ssb3s}*::gfpmutII*) [P_{ssb3s}]. GFP Fluorescence was measured at 510 nm after excitation at 490 nm. Promoter activity was assessed as arbitrary GFP units µg chl*a*$^{-1}$.

4.2.3 Abiotic stress-induced modulation of *ssb* promoter activity

Effect of mitomycin C, γ-irradiation and desiccation stresses on the activity of *ssb* promoters was analysed. Three–day–old recombinant *Anabaena* strains harbouring either pAM1956 (AnpAM) or one of the promoter constructs (designated as P_{ssb1}/P_{ssb2}/P_{ssb3}/P_{ssb3s}) were subjected to either (a) 4 μg mL^{-1} mitomycin C for up to 30 min, (b) 6 kGy of Co^{60} γ-irradiation, or (c) 6 days of desiccation. In case of radiation and desiccation stresses, the stressed cultures and their corresponding controls were washed and inoculated in fresh medium to analyse promoter activity during post-irradiation recovery (PIR) and post-desiccation recovery (PDR). No significant change in the activity of any of the promoters was observed upon exposure to mitomycin C **(Fig. 4.11)**. Immediately upon exposure to 6 kGy of irradiation, the activity of P_{ssb2} and P_{ssb3s} increased by 1.7- and 1.5-fold respectively as compared to unstressed control, while no significant change in activity was observed for P_{ssb1} and P_{ssb3} **(Fig. 4.12A)**. The activity of P_{ssb2} decreased during the later part of PIR i.e., on day 4 and day 6 by 1.8-fold and 2.7-fold respectively **(Fig 4.12A)**. On the other hand, the activity of P_{ssb3s} increased throughout PIR by 1.5-2-fold **(Fig. 4.12A)**. Desiccation stress enhanced the activity of P_{ssb3s} by 1.4-fold, and of P_{ssb2} by 1.18-fold, while that of P_{ssb1} did not show a significant increase **(Fig. 4.12B)**. During recovery from desiccation stress, no significant change in promoter activity was observed for any of the promoters except a 3-fold increase in that of P_{ssb2} on day3 of PDR **(Fig. 4.12B)**.

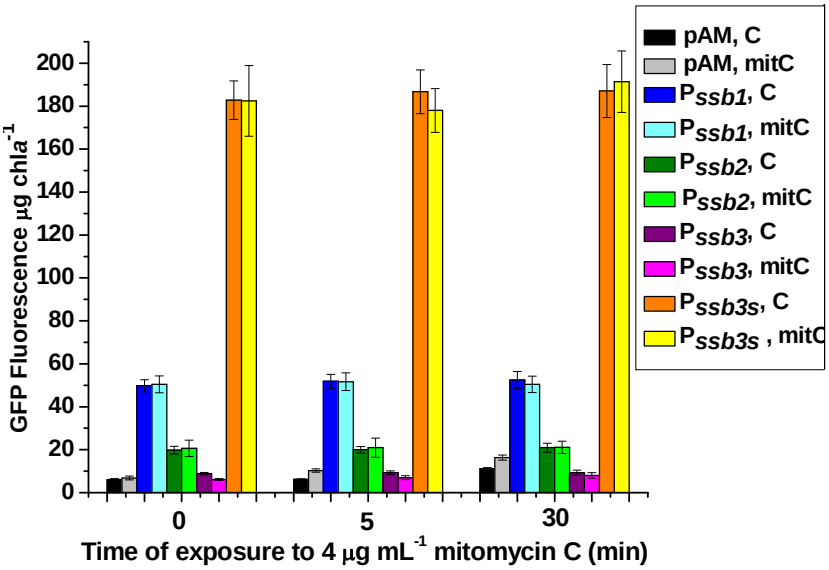

Fig. 4.11 Effect of mitomycin C stress on the activity of *Anabaena ssb* promoters

Three-day-old recombinant *Anabaena* cultures grown under nitrogen-fixing conditions were concentrated to 10 µg chl*a* mL^{-1} and exposed to mitomycin C (4 µg mL^{-1}) for up to 30 min. The activity of *Anabaena ssb* promoters was assessed after 0, 5 and 30 min of exposure. Other details are as described in legend to Fig. 4.10.

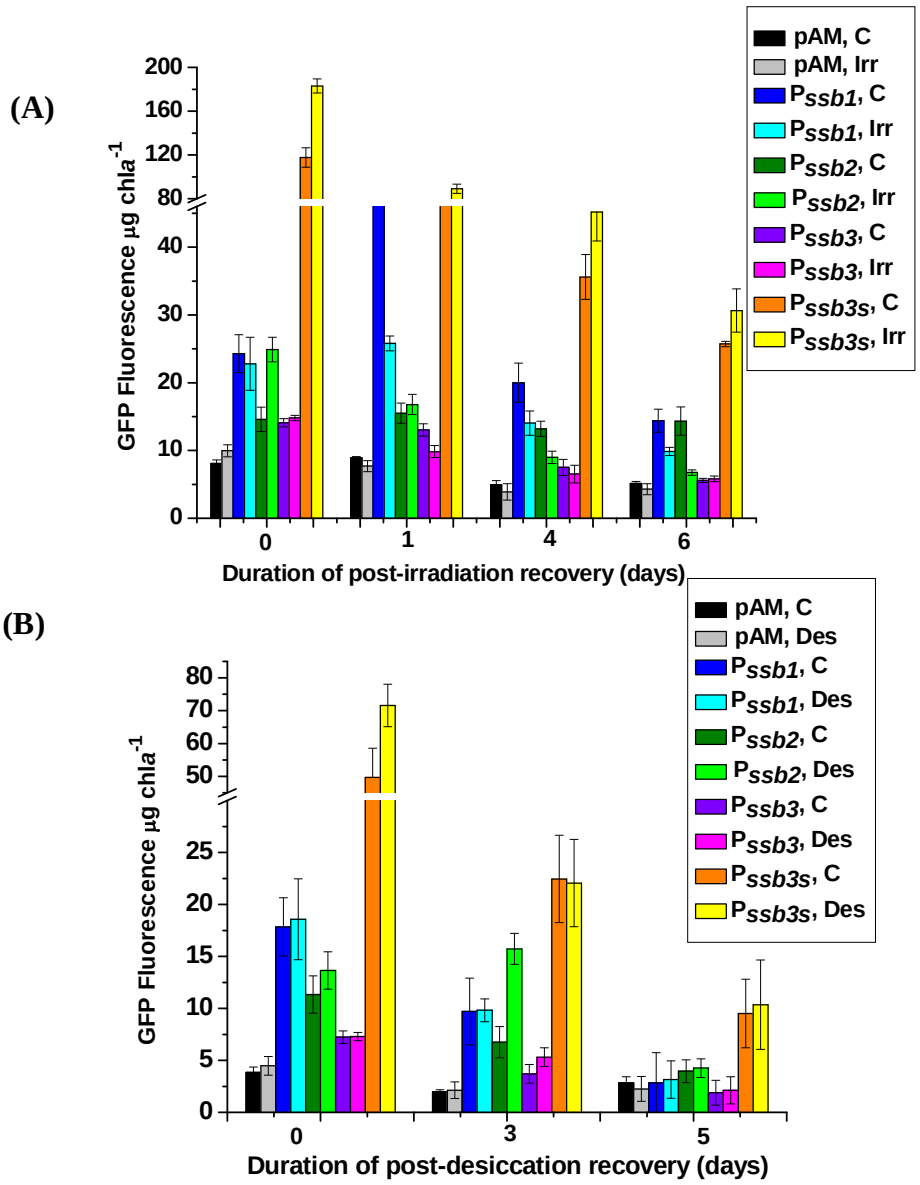

Fig. 4.12 Effect of γ-irradiation and desiccation stresses on the *ssb* promoter activity.

Three-day-old recombinant *Anabaena* cultures grown under nitrogen-fixing conditions were concentrated to 10 µg chl*a* density mL^{-1} and exposed to either (A) 6 kGy of γ-irradiation or (B) 6 days of desiccation. The promoter activity was analysed either immediately after stress or during the recovery period. For recovery after stress, both the unstressed and stressed cultures were washed and inoculated in fresh medium at 1 µg chl*a* mL^{-1} and grown for up to 6 days. Promoter activity was measured after specified days of recovery. Other details are as described in legend to Fig. 4.10.

4.2.4 Expression Profile of SSB proteins in *Anabaena* 7120 in response to different DNA damage inducing stresses

Three-day-old wild type *Anabaena* 7120 cells grown under nitrogen-fixing conditions were subjected to different DNA-damage inducing stresses, namely mitomycin C (0-5 μg mL^{-1}), UV-B stress (0-6 kJ m^{-2}), 6 kGy of γ-irradiation or 6 days of desiccation. Exposure of *Anabaena* cells to mitomycin C (0-5 μg mL^{-1}) resulted in no significant change in expression of any of the three SSB proteins **(Fig. 4.13A)**. The maximum increase was found to be about 1.4-fold for SSB1 and SSB2 after exposure to 3 μg mL^{-1} or 5 μg $mL^{-1}$of mitomycin C **(Fig. 4.13A)**, but the average increase observed over triplicate gels was found to be 1.2 ± 0.22 and 1.2 ± 0.25 respectively **(Table 4.1)**, and hence was not considered significant. Upon exposure to UV-B (0-6 kJ m^{-2} dose), SSB1 and SSB2 expression levels did not change significantly **(Figs. 4.13 B, Table 4.1),** while both the protein bands corresponding to SSB3 exhibited a 1.5-2.2-fold change over the entire range **(Fig. 4.13 B, Table 4.1)**.

Exposure to 6 kGy of γ-irradiation did not result in significant change in expression of *Anabaena* SSB proteins immediately after irradiation **(Fig. 4.14 A, Table 4.2)**. On the other hand, desiccation stress for 6 days increased SSB2 expression by about 1.5-fold (1.3 ± 0.21), decreased that of SSB3 by almost 50% (0.5 ± 0.2), while that of SSB1 remained unchanged **(Fig. 4.14B, Table 4.2)**. During post-irradiation recovery, SSB2 expression enhanced after 24 h, and that of SSB3 increased up to 72 h, while no change was observed for SSB1 **(Fig. 4.15)**.

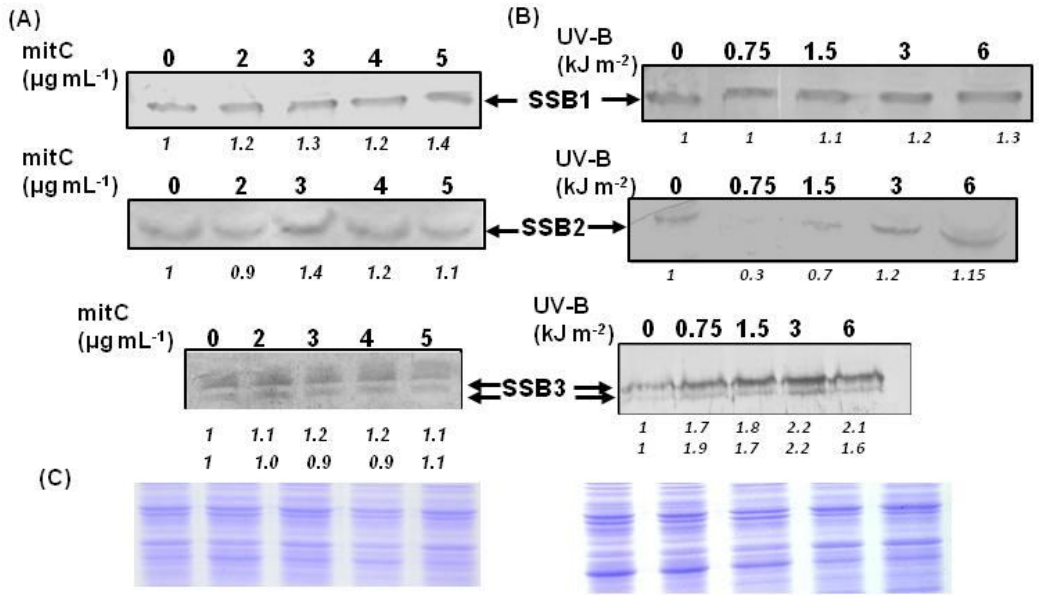

Fig. 4.13 Effect of mitomycin C and UV-B stresses on the expression of *Anabaena* SSB proteins.

Three-day-old *Anabaena* 7120 cultures grown under nitrogen-fixing conditions were concentrated to 10 µg chl*a* mL^{-1} and subjected to either (A) mitomycin C stress (0-5 µg mL^{-1}) for 30 min, or UV-B stress (0-6 kJ m^{-2}). Protein extracts (40 µg mL^{-1}) were electrophoretically separated by 14% SDS-PAGE, electroblotted on to nitrocellulose membrane and probed with polyclonal antibodies raised individually against *Anabaena* SSB1, SSB2 and SSB3 proteins. The cross reacting proteins are indicated by arrows. The fold change in expression of the three proteins with respect to that under control conditions (1st lane) was determined by densitometry and is shown below each gel. (C) Coomassie stained gel of the proteins used for Western blot and immunodetection in (A) and (B).

Table 4.1 Fold-change in expression of SSB proteins in response to mitomycin C and UV-B stresses

Mit C (μg mL^{-1})	**0**	**2**	**3**	**4**	**5**
SSB1	1 ± 0	0.9 ± 0.32	1.1 ± 0.21	1.1 ± 0.15	1.2 ± 0.22
SSB2	1 ± 0	1 ± 0.14	1.2 ± 0.25	1.1 ± 0.1	1 ± 0.13
SSB3U	1 ± 0	1 ± 0.12	0.9 ± 0.05	1 ± 0.1	1 ± 0.15
SSB3L	1 ± 0	0.9 ± 0.1	1 ± 0.13	0.9 ± 0.1	1 ± 0.1
UV-B ($J m^{-2}$)	**0**	**0.75**	**1.5**	**3**	**6**
SSB1	1 ± 0	0.9 ± 0.09	0.95 ± 0.16	1 ± 0.21	1.15 ± 0.18
SSB2	1 ± 0	1.8 ± 0.16	2 ± 0.25	2 ± 0.21	2.1 ± 0.15
SSB3U	1 ± 0	1.8 ± 0.16	2 ± 0.25	2 ± 0.21	2.1 ± 0.15
SSB3L	1 ± 0	2 ± 0.16	1.8 ± 0.1	2 ± 0.22	1.5 ± 0.15

Table 4.2 Fold-change in expression of SSB proteins in response to γ-irradiation and desiccation stresses

Protein	**γ-irradiation stress**		**desiccation stresses**	
	0 kGy	**6 kGy**	**Humid chamber**	**6 days of desiccation**
SSB1	1 ± 0	0.8 ± 0.22	1 ± 0	0.95 ± 0.17
SSB2	1 ± 0	1 ± 0.16	1 ± 0	1.3 ± 0.21
SSB3U	1 ± 0	1.1 ± 0.15	1 ± 0	0.5 ± 0.2

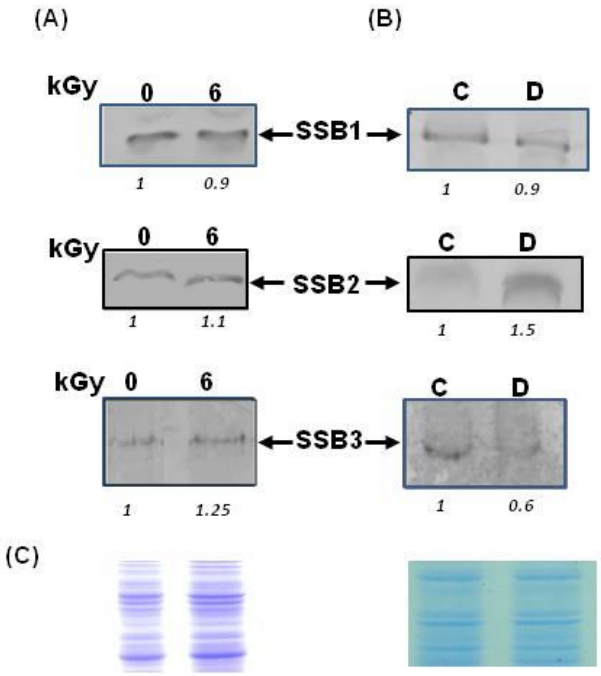

Fig. 4.14 Effect of γ-irradiation and desiccation stresses on the expression of *Anabaena* SSB proteins

Three-day-old *Anabaena* 7120 cultures grown under nitrogen-fixing conditions were concentrated to 10 μg chl*a* density mL^{-1} and subjected to either (A) 6 kGy of ^{60}Co γ-irradiation, or (B) 6 days of desiccation. The cross-reacting *Anabaena* SSB proteins are indicated with arrows. (C) Coomassie stained gel of the proteins used for Western blot and immunodetection in (A) and (B).

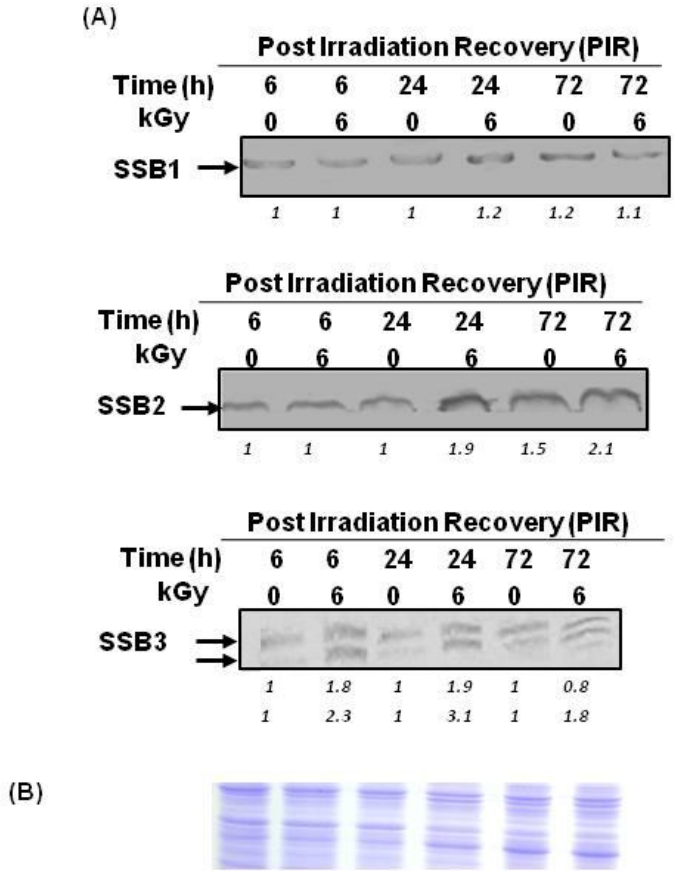

Fig. 4.15 Expression of *Anabaena* SSB proteins during recovery from radiation stress

(A) Immunodetection of SSB proteins during post-irradiation recovery (PIR). Three-day-old *Anabaena* 7120 cultures grown under nitrogen-fixing conditions were concentrated to 10 µg chl*a* mL^{-1} and subjected to 6 kGy of ^{60}Co γ-irradiation. The control or irradiated cultures were washed and inoculated in fresh medium at 1 µg chl*a* density mL^{-1} and allowed to grow under control conditions. Other details are as described in legend to Fig. 4.13. (B) Coomassie stained gel of the proteins used for Western blot and immunodetection in (A).

4.2.5 Interaction of *Anabaena* LexA with *ssb* promoters

Bioinformatic analysis of the upstream region of *ssb1* indicates the presence of a putative LexA binding box (**AGTACTTATGTACT)** having a 5 bp palindromic sequence separated by 4 bases **(Fig. 4.6A)**. In case of *ssb2* promoter, the putative LexA box (**ACTCATAAGT**) **(Fig. 4.6B)**, comprised of a 3 bp palindromic sequence separated by 4 bases. However, the orientation of the two palindromic arms in the putative LexA box of P_{ssb2} was opposite of that in P_{ssb1}. No LexA-box-like element was found in case of *ssb3* promoter **(Fig. 4.6C)**.

The presence of LexA box-like element is indicative of possible regulation of the promoters by LexA. The binding of purified native *Anabaena* LexA to the three *ssb* promoters was analysed by electrophoretic mobility shift assay (EMSA) **(Fig. 4.16)**. At a fixed concentration of P_{ssb1}, the mobility of the fragment was found to be retarded with increasing concentration of *Anabaena* LexA used (0.25-4.5 µg). At the lowest concentration tested (0.25 µg of LexA), a minor shift in the mobility of P_{ssb1} was observed **(Fig. 4.16A)**, while upon increasing the concentration of LexA further, most of the P_{ssb1}-LexA complex was detected in the well **(Fig. 4.16A)**. The binding of LexA (0.5 µg) to P_{ssb2} and P_{ssb3} was also assessed. While a complete retardation of P_{ssb2} was observed in the presence of LexA, similar to that observed for P_{ssb1}, there was no change in mobility of P_{ssb3} **(Fig. 4.16B)**. Thus, the binding of *Anabaena* LexA was specific to the LexA box present in P_{ssb1} and P_{ssb2}.

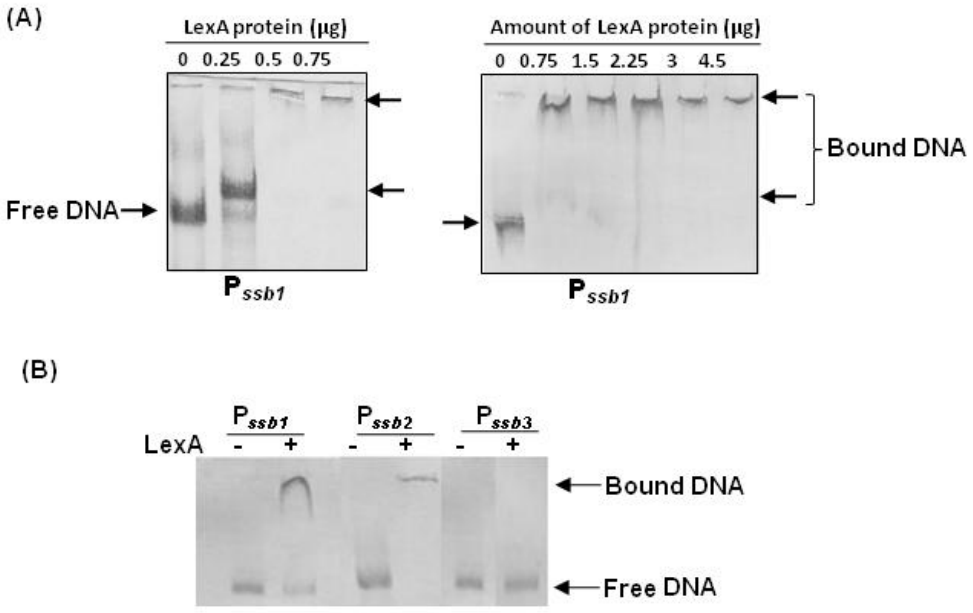

Fig. 4.16 Binding of *Anabaena* LexA to *ssb* promoters

Electro-mobility shift assay of DIG-labelled (A) *ssb1* promoter in the presence of increasing concentration of *Anabaena* LexA protein. (B) *ssb1*, *ssb2* and *ssb3* promoter fragments in the presence of 0.5 µg LexA protein. Following *in solution* interaction, the assay mix was separated on 6% non-denaturing PAGE in 1X TBE and electroblotted on to nylon membrane. The blot was treated with anti-DIG antibody followed by colour development using NBT-BCIP. The free DNA and bound DNA are indicated by arrows.

4.3 DISCUSSION

In general, bacterial *ssb* gene is present adjacent to other DNA repair/recombination genes, such as *uvrA*, *recF*, at times as part of the same operon (11). However, in case of *Anabaena* 7120 none of the *ssb* genes are located adjacent to identified DNA repair genes **(Figs. 4.1-4.3)**, and this organisation is unique to *Anabaena* 7120, justifying its grouping of *ssb* genes in Group IV (105).

In spite of *ssb1* and *ssb2* coding for truncated SSB proteins, both the genes along with *ssb3*, which codes for full length SSB were expressed under normal growth conditions both at the transcript **(Figs 4.4 and 4.5)** and protein level **(Figs. 4.13-4.15)**. This indicated that all three *ssb* genes of *Anabaena* are functional and are possibly required for cellular processes under normal growth conditions. Each of these *ssb* genes was transcribed by its own promoter, as confirmed by bioinformatic and RT-PCR analysis **(Figs. 4.1-4.5)**, irrespective of whether they existed as a monocistronic gene as is the case with *ssb1*, or as part of a bicistronic operon as observed for *ssb3*.

Promoter analysis of the three *ssb* genes indicated that all are likely to be regulated by either *cis*- or *trans*-acting factors. All three promoters possessed a typical -10 and -35–like regions **(Fig. 4.6)** indicative of the involvement of a vegetative promoter for transcription. The presence of LexA box-like sequence upstream of the two truncated *ssb* genes (*ssb1* and *ssb2*) **(Fig. 4.6)**, suggested possible regulation by LexA as has been observed for other bacteria (143). The binding of *Anabaena* LexA specifically to *ssb1* and *ssb2* promoters having LexA box, but not *ssb3* which lacks the LexA box **(Fig. 4.16)** suggested possible regulation of *ssb1* and *ssb2* by LexA. The binding of LexA was not affected by the orientation of the two palindromic arms of the LexA-box, since LexA

bound both *ssb1* and *ssb2* promoters, which differ in the length as well as orientation of the two palindromic arms **(Figs. 4.6A and B)**. The *ssb3* promoter is negatively regulated by an AT-rich region located about 150 bp upstream of the start codon **(Fig. 4.6C)**. The repression of expression from the promoter in the presence of the AT-rich element in *Anabaena*, but not in *E. coli* **(Fig. 4.9)**, suggested the possible involvement of a *trans*-acting protein, specific to *Anabaena*. Preliminary efforts to identify such a protein were, however, not successful. The identified AT-rich region did not bear any homology to RDRM sequence, known to play a role in regulation of *ssb* expression in *D. radiodurans* (119).

The regulation of the *ssb* promoters in response to exposure to different abiotic stresses was at the transcriptional level, as confirmed by significant agreement in stress-induced modulation in *ssb* promoter activity **(Figs. 4.10-4.12)** and expression of the corresponding SSB protein **(Figs. 4.13-4.15)**. Exposure to stresses which cause formation of DNA adducts, such as mitomycin C and UV-B radiation had no effect on the expression of the truncated *ssb* genes, *ssb1* and *ssb2*, both in terms of change in promoter activity and protein expression **(Figs. 4.10 and 4.13)**, while that of *ssb3* was enhanced in response to UV-B stress **(Fig. 4.13)**. In general, either or both the stresses are known to induce expression of bacterial *ssb* genes (11, 101, 119). On the other hand, exposure to stresses which caused single strand and double strand breaks, such as γ-radiation and desiccation modulated the expression of all three *ssb* genes, either immediately after stress or during recovery from the above stresses **(Figs. 4.11, 4.12, 4.14 and 4.15)**. Modulation of expression of *ssb* genes in response to γ-radiation has been reported in case of *D. radiodurans*, and was speculated to be through the RDRM sequence (119).

The modulation of expression of *ssb3* in response to most DNA-damage inducing stresses suggested that SSB3 is probably playing a crucial role in DNA repair process, irrespective of the kind of repair. The presence of acidic tail, only in SSB3, would enable its interaction with other DNA repair proteins and thus making a functional DNA repair unit.

CHAPTER 5

Possible physiological roles of SSB proteins

5.1 INTRODUCTION

In bacteria, SSB proteins are involved in several important processes such as DNA replication, recombination, repair etc. (11). They are vital for cell survival and deletion of the *ssb* genes is lethal (39). So, several point mutants and temperature sensitive mutants were generated in *E. coli*. Most *ssb* mutants are found to be temperature sensitive for DNA replication (11). They are also defective in a variety of DNA repair processes, UV sensitive, defective in RecA induction, Weigle reactivation, and mutagenesis, and also exhibited excessive post-irradiation DNA degradation (11). Therefore, all aspects of DNA metabolism, be it replication, repair or recombination, are affected upon mutation of *ssb*. Since most cyanobacteria including *Anabaena* 7120 have two or more annotated *ssb*-like genes, it is speculated that individual *ssb* gene mutants of *Anabaena* may survive, since lack of one of the SSB proteins could possibly be compensated by the presence of other two SSB proteins.

SSBs are involved in coating of ssDNA and prevent formation of ds duplex, and thereby overexpression of the protein could result in disruption of the ds DNA and thus prevent/inhibit several processes involving DNA. However, SSB overexpressing bacteria are viable. In *E. coli*, overexpression of SSB leads to a decrease in the basal level of the repressor LexA, which in turn results in increased, but differential expression of the LexA-controlled genes (100). It increases the efficiency of excision repair, but reduces that of post-replication recombinational repair due to increasing formation of short nucleofilament complexes (100). DNA replication is also affected to some extent upon overexpression of SSB in *E. coli* (100).

5.2 RESULTS

5.2.1 Generation of *ssb* mutant strains of *Anabaena*

Recombinant *Anabaena* strains wherein the *ssb* gene is replaced by *nptII* cassette was obtained by electroporating recombinant plasmids, namely pBS*ssb*1M, pBS*ssb2*M and pBS*ssb3*M. The generation of these plasmid constructs required a four step approach as shown in **Fig. 5.1**.

(i) The first step involved cloning of ~1 kb region upstream of the *ssb* gene in pBluescript II (pBS) vector. This corresponded to the region 19-871 bp upstream of *ssb1* gene, 71-975 bp upstream of *ssb2* gene and from 28-959 bp upstream of *ssb3* gene. These regions were PCR amplified from the chromosomal DNA of *Anabaena* 7120 using *ssb*(1/2/3)mut1Fwd (having *Eco*RI restriction site) and *ssb*(1/2/3) mut2Rev (having *Bam*HI restriction site) primers **(Table 2.3) (Step A, Fig. 5.1)**. The resulting amplicons for the three genes designated as *ssb1*Up, *ssb2*Up and *ssb3*Up were digested with *Eco*RI and *Bam*HI restriction enzymes, ligated to pBluescript (pBS) at *Eco*RI/*Bam*HI sites **(Step B, Fig. 5.1)** and transformed into *E. coli* DH5α cells. The recombinant clones were confirmed by colony PCR using respective primers and designated as pBS*ssb1*Up, pBS*ssb2*Up and pBS*ssb3*Up respectively. Restriction digestion of these plasmids with *Eco*RI and *Bam*HI enzymes resulted in the release of 852 bp *ssb1*Up, 904 bp *ssb2*Up and 931 bp *ssb3*Up fragments in addition to the 3 kb pBS vector DNA **(Figs. 5.2 A-C)**.

(ii) The second step involved cloning of ~1 kb region downstream to the *ssb* gene in pBS vector. This corresponded to region from 25 to 881 bp downstream of *ssb1*, from 12-973 bp downstream of *ssb2* and from 31-943 bp downstream of *ssb3*. The fragments were PCR amplified from *Anabaena* 7120 genomic DNA using *ssb*(1/2/3)mut3Fwd (having

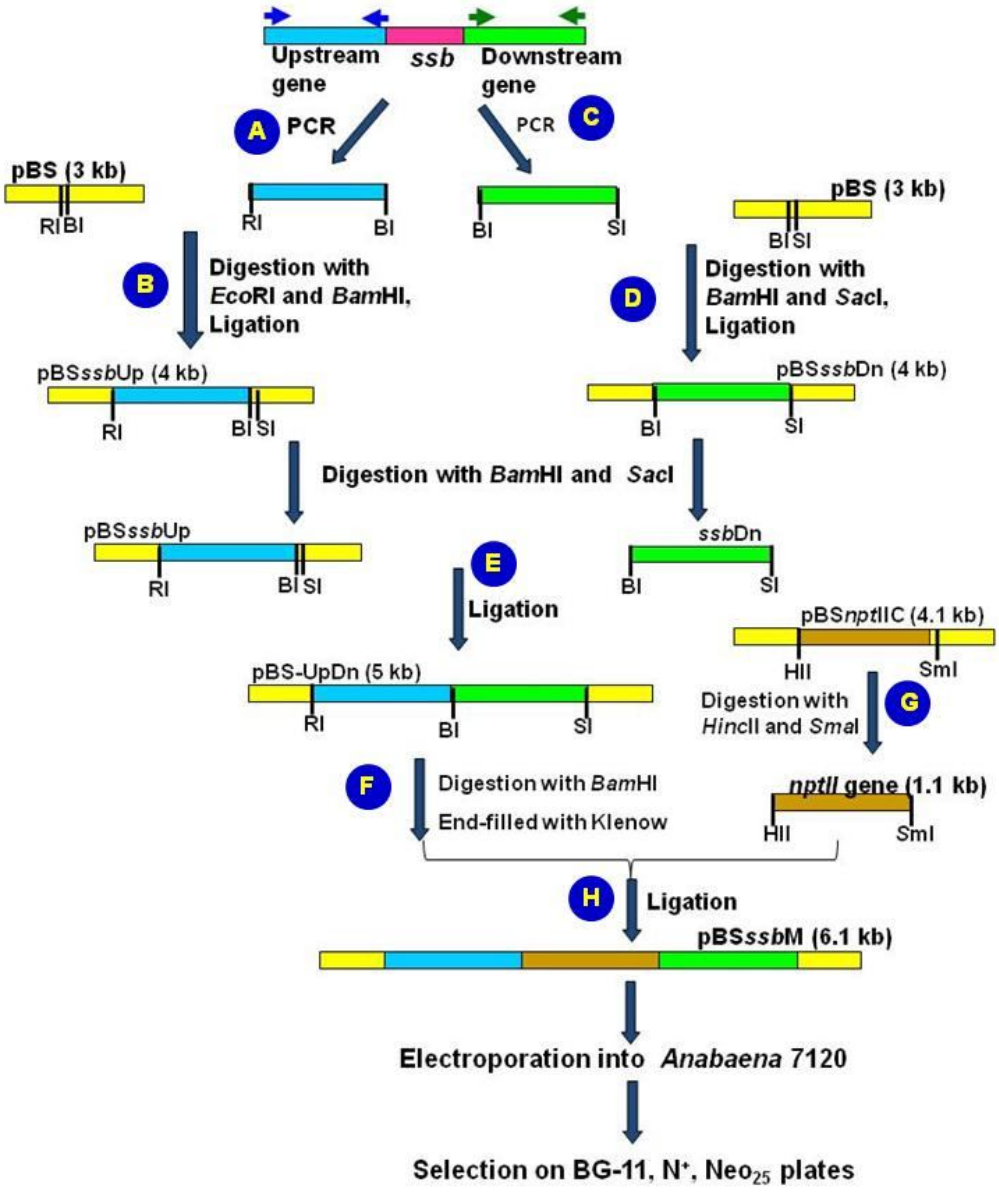

Fig. 5.1 Schematic representation of the steps involved in the generation of *Anabaena ssb* mutants.

All genes and plasmids are shown as rectangular boxes of different colours as indicated. The different restriction sites indicated are: BI: *Bam*HI, HII: *Hinc*II, RI: *Eco*RI, SI: *Sac*I and SmI: *Sma*I.

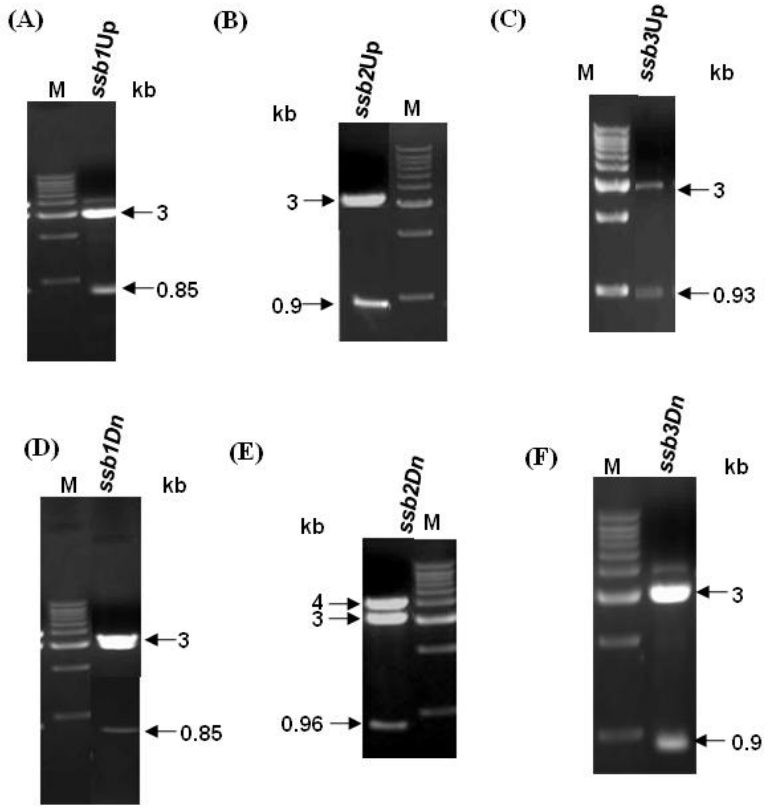

Fig. 5.2 Cloning of the regions upstream and downstream of the *ssb* genes of *Anabaena* 7120.

(A-C) Restriction digestion of (A) pBS*ssb1U*p, (B) pBS*ssb2U*p, and (C) pBS*ssb3U*p plasmids with *Eco*RI and *Bam*HI. (D-F) Restriction digestion of (D) pBS*ssb1Dn*, (F) pBS*ssb2Dn*, and (F) pBS*ssb3Dn* plasmids with *Bam*HI and. *Sac*I. The sizes of different DNA fragments are indicated. M represents 1 kb DNA ladder. Other details are as described in legend to Fig. 3.5.

*Bam*HI restriction site) and *ssb*(1/2/3)mut4Rev (having *Sac*I restriction site) primers **(Table 2.3) (Step C, Fig. 5.1A)**. The resulting amplicons designated as *ssb1*Dn, *ssb2*Dn and *ssb3*Dn and of 856 bp, 961 bp and 912 bp respectively were digested with *Bam*HI and *Sac*I restriction enzymes, ligated to pBS vector at *Bam*HI/*Sac*I sites **(Step D, Fig. 5.1)** and transformed into *E. coli* DH5α cells. Positive clones were confirmed by PCR amplification and restriction digestion with *Bam*HI and *Sac*I enzymes which resulted in the release of 0.85 kb **(Fig. 5.2D)**, 0.96 kb **(Fig. 5.2E)** and 0.91 kb **(Fig. 5.2F)** inserts along with the 3 kb pBS vector DNA. The corresponding plasmids were designated as pBS*ssb1*Dn, pBS*ssb2*Dn and pBS*ssb3*Dn respectively. Sequence of all the amplicons was confirmed by DNA sequencing.

(iii) The third step involved ligation of 856 bp, 961 bp and 912 bp *Bam*HI-*Sac*I fragments from pBS*ssb1*Dn, pBS*ssb2*Dn and pBS*ssb3*Dn respectively (serving as an insert) to 3.85-3.9 kb *Bam*HI-*Sac*I digested pBS*ssb1*Up, pBS*ssb2*Up and pBS*ssb3*Up plasmids respectively (serving as vector) and transformation into *E. coli* DH5α cells **(Step E, Fig. 5.1A)**. The resulting constructs were confirmed by PCR amplification using *ssb*(1/2/3)mut1Fwd and *ssb*(1/2/3)mut4Rev primers which yielded a 1.6 kb amplicon for *ssb1* and 2 kb amplification product for *ssb2* and *ssb3*. Restriction digestion of (a) pBS*ssb1*UpDn with *Bam*HI and *Sac*I resulted in the release of 1 kb and 4 kb fragments **(Fig. 5.3A)**, (b) pBS*ssb2*UpDn with *Eco*RI in a 5 kb fragment **(Fig. 5.3B)**, and (c) pBS*ssb3* UpDn with *Eco*RI and *Sac*I in 2 kb and 3 kb fragments **(Fig. 5.3C)**, confirming the presence of insert.

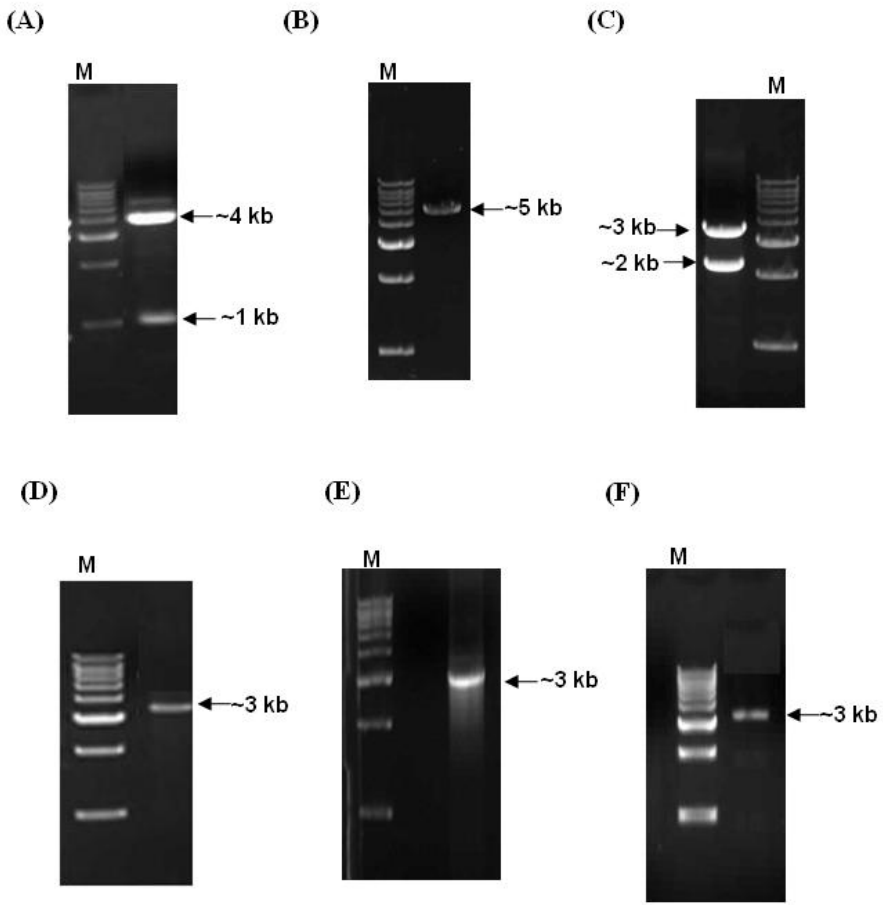

Fig. 5.3 Cloning and confirmation of the *ssb* mutant plasmid constructs.

(A-C) Restriction digestion of plasmids (A) pBS*ssb1*UpDn with *Bam*HI and *Sac*I, (B) pBS*ssb2*UpDn with *Eco*RI and (C) pBS*ssb3*UpDn with *Eco*RI and *Sac*I. (D-F) PCR amplification of (D) pBS*ssb1*M, (E) pBS*ssb2*M and (F) pBS*ssb3*M plasmids with Fwd primer of upstream region and Rev primer of the downstream region for each *ssb* respectively. The sizes of different DNA fragments are indicated. M represents 1 kb DNA ladder. Other details are as described in legend to Fig. 3.5.

(iv) In the fourth step, the plasmids pBS*ssb*(1/2/3)UpDn clones were linearised with *Bam*HI restriction enzyme, the sticky end filled using dNTPs and Klenow enzyme **(Step F, Fig. 5.1)** to generate blunt ends and ligated with 1.1 kb *nptII* cassette **(Step H, Fig. 5.1)** obtained by *Hinc*II-*Sma*I digestion of the plasmid, pBS*nptII*C **(Step G, Fig. 5.1)**. PCR amplification of the resulting plasmid constructs with *ssb*(1/2/3)mut1Fwd and ssb(1/2/3)mut4Rev primers resulted in an amplicon of ~3 kb fragment **(Fig. 5.3 D-F)**.The resulting constructs were designated as pBS*ssb*1M, pBS*ssb*2M and pBS*ssb*3M respectively.

These plasmids were individually introduced into *Anabaena* 7120 by electroporation at 1200 V with a 5 msec pulse, and Neo^r recombinants selected on BG-11, N^+ Neo_{10} plates by repeated subculturing. A few Neo^r recombinant colonies were obtained for each of the individual *ssb* gene mutants, designated as An*ssb1*$^-$, An*ssb2*$^-$ and An*ssb3*$^-$ **(Table 2.5)**. The vector control AnpAM **(Table 2.5)** appeared as healthy green filaments under bright filed microscope **(Fig. 5.4A)**, as against unhealthy and pale appearance of all the three *ssb* mutant strains of *Anabaena* **(Fig. 5.4 B-D)**. Most of the An*ssb1*$^-$ mutant colonies were either colourless and lysed, or green and hollow when visualised by bright light microscopy **(Fig. 5.4B)**, while the An*ssb2*$^-$ mutants appeared as short filaments with misaligned cells **(Fig. 5.4C)**. The An*ssb3*$^-$ mutants were green, but most of the filaments had only 3-4 cells or in some cases only single cells **(Fig. 5.4D)**. None of the mutants were able to grow in liquid medium. *Anabaena* filaments require minimum filament length of 6-8 cells for subsequent division and growth. Thus, for this reason An*ssb3*$^-$ cells, in spite of being green, did not grow any further.

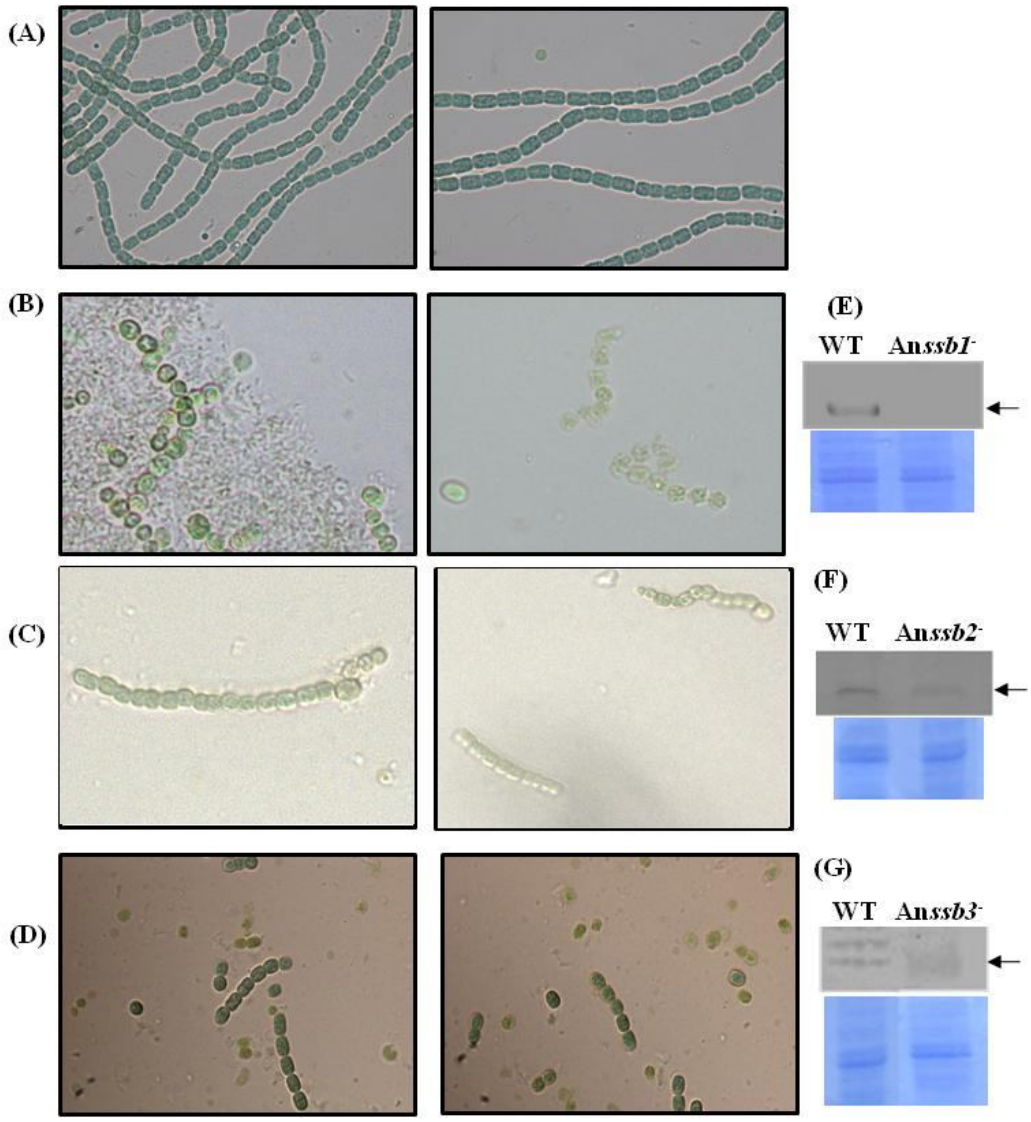

Fig. 5.4 Recombinant *Anabaena ssb* mutant strains.

(A-D) Bright light microphotographs (600 X magnification) of recombinant *Anabaena* strains (A) AnpAM, (B) An*ssb*$^{-}$, (C) An*ssb2*$^{-}$ and (D) An*ssb3*$^{-}$. (E-F) Immunodetection of (E) SSB1 in *Anabaena* 7120 (WT) and An*ssb1*$^{-}$, (F) SSB2 in WT and An*ssb2*$^{-}$ and (G) SSB3 in WT and An*ssb3*$^{-}$ cells. The corresponding Coomassie stained gel is shown below each western blot. Other details are described in legend to Fig. 4.13.

The confirmation of cell death due to mutation was carried out by comparing the levels of the corresponding SSB protein in the mutant and wild type cells. Colonies scrapped from agar plates were used for extraction of protein followed by western blotting and immunodetection with their corresponding specific antibodies. The absence of SSB1 in An*ssb1*$^{-}$ **(Fig. 5.4 E)**, SSB2 in An*ssb2*$^{-}$ **(Fig. 5.4 F)** and SSB3 in An*ssb3*$^{-}$ **(Fig. 5.4 G)** and presence of all three proteins in wild type *Anabaena* 7120 **(Figs. 5.4 E-G)** confirmed the corresponding mutation in the *ssb* genes.

5.2.2 Generation of recombinant *Anabaena* strains overexpressing SSB proteins

The strategy for overexpression of SSB in *Anabaena* 7120 involved introduction of *ssb* in *trans* under the control of a constitutive promoter in addition to the wild type copy using a vector that can replicate in *Anabaena* 7120. The *Nde*I/*Bam*HI fragment corresponding to the complete *ssb* gene was released from 'pET*ssb*(1/2/3)' **(Table 2.4)** by restriction digestion **(Step A, Fig. 5.5)** and ligated to pFPN vector **(Table 2.4)** digested with *Nde*I and *Bam*HI restriction endonucleases **(Step B, Fig. 5.5)** downstream of the *psbA*1 promoter. The transcription of the *ssb* gene would thus be under the control of *psbA*1 promoter. The resulting constructs were designated as 'pFPN*ssb*(1/2/3)' **(Table 2.4)**. The presence of insert in the above plasmid constructs was confirmed by restriction digestion with *Nde*I/*Bam*HI enzymes. In addition to the release of ~5 kb pFPN vector, an insert of 0.36 kb, 0.38 kb and 0.55 kb corresponding to *ssb1*, *ssb2* and *ssb3* respectively were also released **(Fig. 5.6A)**.

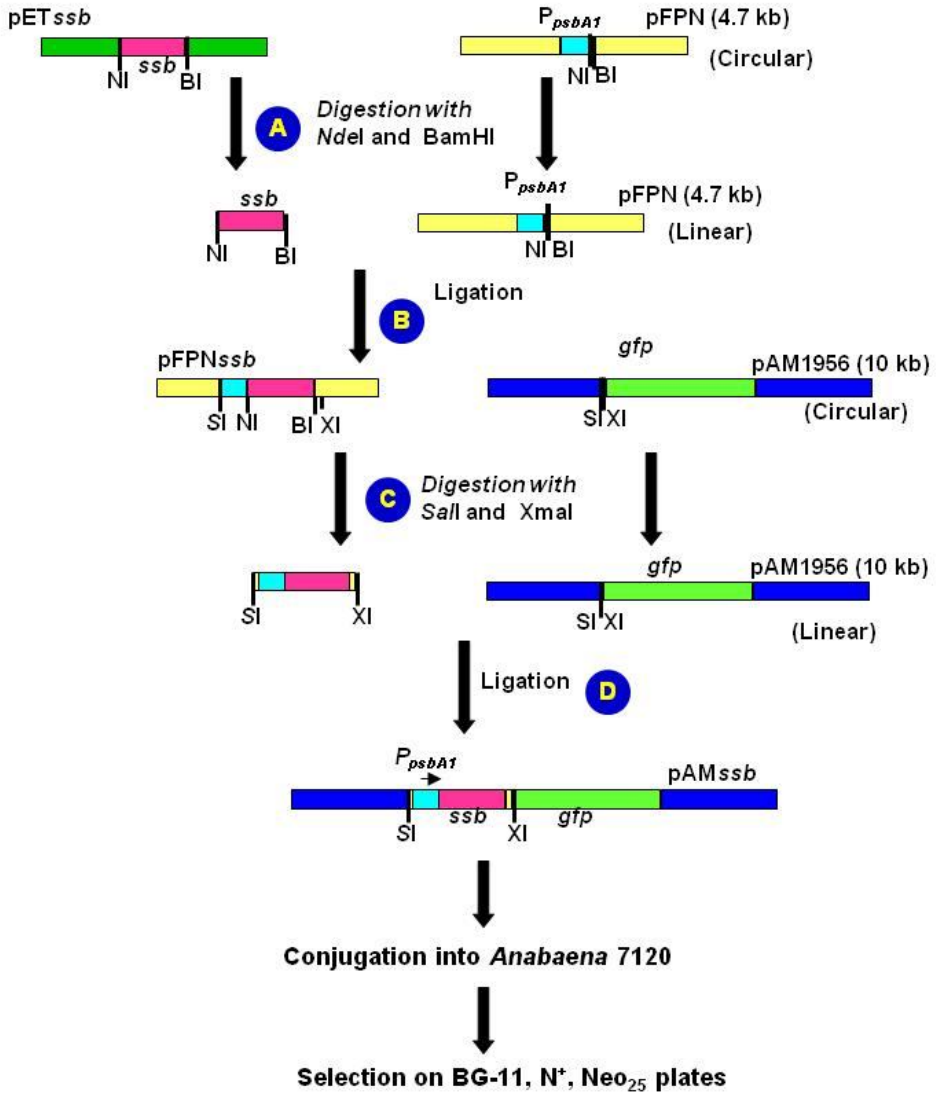

Fig. 5.5 Schematic representation of the steps involved in the generation of *Anabaena* SSB overexpressing strains.

All genes and plasmids are shown as rectangular boxes of different colours as indicated. The different restriction sites indicated are: BI: *Bam*HI, NI: *Nde*I, SI: *Sal*I and XI: *Xma*I.

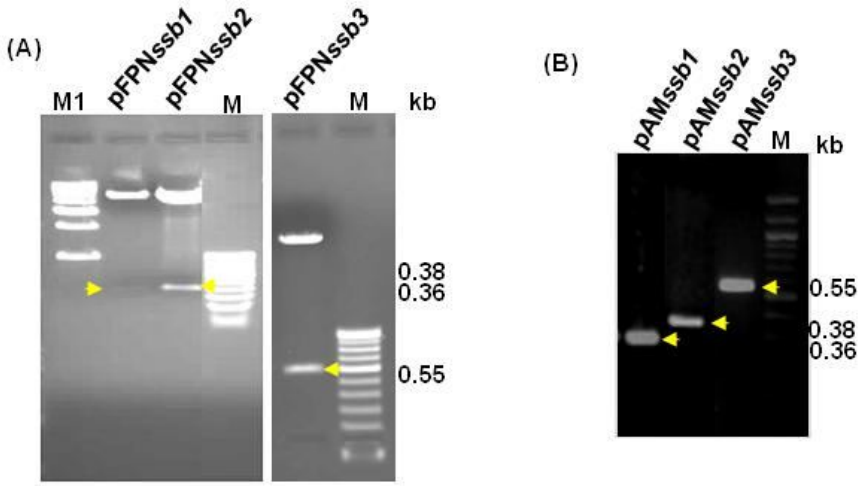

Fig. 5.6 Cloning and confirmation of the SSB overexpression plasmid constructs.

(A) Restriction digestion of plasmids, pFPN*ssb1*, pFPN*ssb2* and pFPN*ssb3* with *Nde*I and *Bam*HI restriction enzymes. (B) PCR amplification of pAM*ssb1*, pAM*ssb2* and pAM*ssb3* plasmid DNA with gene specific primers. The sizes of different DNA fragments are indicated. M1 represents 1 kb DNA ladder and M, 100 bp DNA ladder. Other details are as described in legend to Fig. 3.5

The *ssb* gene along with *psbA*1 promoter (~180 bp) was excised from the constructs 'pFPN*ssb*(1*/2/3*)' using *Sal*I/*Xma*I restriction enzymes **(Step C, Fig. 5.5)** and ligated to promoterless pAM1956 vector **(Table 2.4)**, at the same set of restriction sites *Sal*I/*Xma*I, **(Step D, Fig. 5.5)**. The resulting plasmid constructs were designated as 'pAM*ssb*(1/2/3)' **(Table 2.4)**. The cloning of the corresponding gene in the plasmid constructs was confirmed by PCR amplification using gene specific primers. Amplicons of 0.36 kb, 0.38 kb and 0.55 kb were obtained by PCR amplification of pAM*ssb1*, pAM*ssb2* and pAM*ssb3* respectively **(Fig. 5.6B)**. In pAM*ssb*(1/2/3) plasmid constructs, the *ssb* gene is transcriptionally fused with *gfpmutII* (coding for Green Fluorescence Protein) of pAM1956. As a result, the *ssb*(*1/2/3*) and *gfpmutII* genes are co-transcribed from *psbA*1 promoter. Hence, the expression of GFP in the recombinant cells can be used for fluorescence microscopy based monitoring cloning as well as extent of segregation.

The plasmid constructs, pAM*ssb*1, pAM*ssb*2 and pAM*ssb3* were individually introduced into *Anabaena* 7120 by tri-parental conjugation and the exconjugants selected on BG-11, N^+, Neo_{25} plates were screened for GFP expression by observing them under fluorescence microscope using excitation wavelength of 470 nm and emission at 508 nm. The wild type *Anabaena* 7120 will exhibit red fluorescence due to the presence of chlorophyll. On the other hand, the recombinant *Anabaena* strains expressing GFP will exhibit orange to green fluorescence. The variation in colour arises since the green fluorescence due to GFP has to mask the red fluorescence due to the

presence of chlorophyll in these cells, and the amount of GFP expressed would determine the extent of masking and hence the colour of fluorescence.

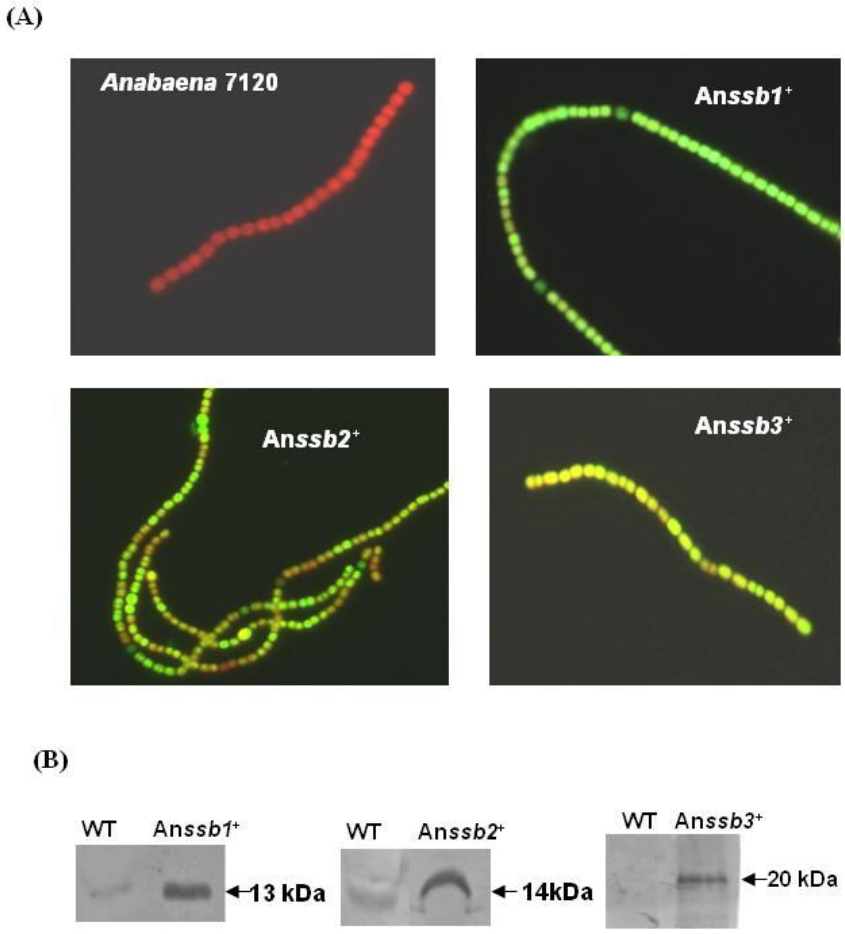

Fig. 5.7 Recombinant *Anabaena* strains overexpressing SSB protein.

(A) Fluoromicrophotographs (600 X magnification) of wild type (*Anabaena* 7120) and recombinant *Anabaena* strains (An*ssb1*$^{+}$, An*ssb2*$^{+}$ and An*ssb3*$^{+}$). Other details are as described in legend to Fig. 4.9. Immunodetection of the overexpressed SSB in wild type (WT) and the recombinant *Anabaena* strains. The overexpressed SSB is indicated with an arrow along with its molecular mass. Other details are described in legend to Fig. 4.13.

The recombinant *Anabaena* strains were subjected to further segregation by repeated streaking on plates or by subculturing in liquid media, followed by mild sonication and plating. The different recombinant strains, An*ssb1*$^{+}$, An*ssb2*$^{+}$, An*ssb3*$^{+}$ harbouring the plasmids pAM*ssb1*, pAM*ssb2* and pAM*ssb3* respectively, co-expressing GFP exhibited green fluorescence as against red fluorescence observed in wild type, *Anabaena* 7120 **(Fig. 5.7A)**. Western blotting and immunodetection analysis of the protein extracts from the recombinant *Anabaena* strains, An*ssb1*$^{+}$, An*ssb2*$^{+}$, An*ssb3*$^{+}$ with their respective antibodies revealed higher levels of the corresponding SSB proteins compared to wild type *Anabaena* 7120 **(Fig. 5.7B)**, thereby confirming overexpression of the corresponding SSB proteins.

5.2.3 Effect of SSB overexpression on normal growth and stress tolerance of *Anabaena*

Growth of the recombinant *Anabaena* strains overexpressing SSB proteins, compared to vector control, AnpAM **(Table 2.4)** was monitored in terms of chlorophyll *a* content over a 7-day period. The increase in chlorophyll *a* content was found to be similar for all the four recombinant *Anabaena* strains **(Fig. 5.8)**, indicating that SSB overexpression had no effect on normal cell growth or physiology of *Anabaena*. Their effect on the ability of *Anabaena* to tolerate different DNA damage inducing stresses was tested. The different stresses applied were mitomycin C and UV-B, both of which cause DNA adduct formation, and γ-irradiation and desiccation stresses which induce ss and ds breaks in DNA.

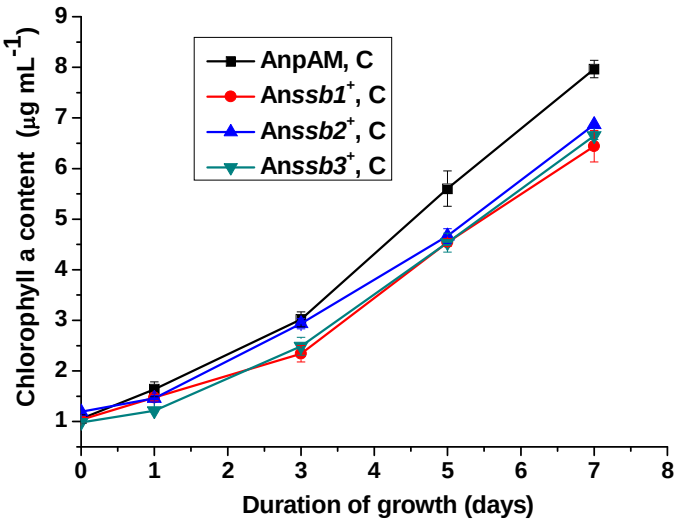

Fig. 5.8 Growth of recombinant *Anabaena* strains overexpressing SSB protein under nitrogen-fixing conditions

Three-day-old nitrogen-fixing cultures of recombinant *Anabaena* strains, AnpAM (harbouring the empty plasmid pAM1956), An*ssb1*$^{+}$, An*ssb2*$^{+}$ and An*ssb3*$^{+}$ overexpressing SSB1, SSB2 and SSB3 respectively were inoculated in fresh BG-11, N^{-}, Neo_{25} medium at 1 µg chl*a* density mL^{-1} and grown under stationary conditions with constant illumination for 7 days. Growth was measured in terms of chlorophyll *a* content.

(a) Mitomycin C Stress

Three-day-old nitrogen-fixing cultures of recombinant *Anabaena* strains, AnpAM, An*ssb1*$^{+}$, An*ssb2*$^{+}$ and An*ssb3*$^{+}$ (10 μg chl*a* density mL^{-1}) were exposed to different doses of mitomycin C (0, 2 and 4 μg mL^{-1}) for 30 min and survival assessed in terms of colony forming units (CFUs). Exposure to 2 or 4 μg mitomycin C mL^{-1} for 30 min, decreased the survival of AnpAM cells to about 64.5% and 61% respectively **(Fig. 5.9A)**. Overexpression of either of the SSB proteins enhanced the survival of *Anabaena* cells to mitomycin C, but the extent of increase in survival varied. Overexpression of SSB1 increased the survival to 84.7% and 125.7 % respectively after exposure to 2 μg mL^{-1} and 4 μg mL^{-1} mit C respectively **(Fig. 5.9A)**. Overexpression of SSB2, on the other hand, resulted in 83.2% and 72.6% survival after 30 min exposure to 2 μg mL^{-1} and 4 μg mL^{-1} respectively, while that for An*ssb3*$^{+}$ was 103% and 84.4% respectively **(Fig. 5.9A)**. All the four recombinant strains undergo fragmentation upon exposure to 4 μg mL^{-1} for 30 min, resulting in about 20-30% shorter filaments than that under unstressed conditions and thus could give rise to higher colony count than the unstressed control cultures. Since, the fragmentation is independent of the protein being overexpressed, it can be safely concluded that SSB1 overexpression offers maximum protection against exposure to 4 μg mL^{-1} mitomycin C.

(b) UV-B stress

Survival of AnpAM cells decreased to 48.7% upon exposure to 0.75 kJ m^{-2} of UV-B, which further decreased to 11.6% at 1.5 kJ m^{-2} **(Fig. 5.9B)**. These cells could not

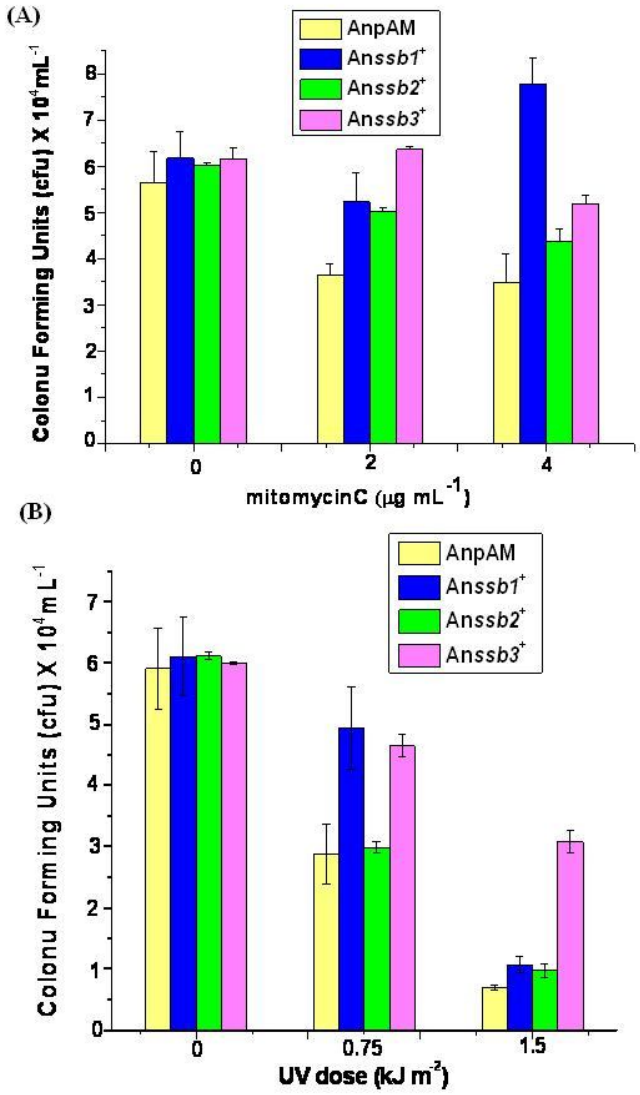

Fig. 5.9 Effect of SSB overexpression on tolerance of *Anabaena* to stresses which induce DNA adducts formation.

Three-day-old recombinant *Anabaena* cultures were concentrated to 10 µg chl*a* density mL^{-1} and exposed to either (A) different concentrations of mitomycin C for 30 min or (B) different doses of UV-B (dose rate 5 J $m^{-2}s^{-1}$). An aliquot of the culture was plated on to BG-11, N-, Neo_{25} agar plates and incubate under constant illumination for 10 days. Colonies were counted at the end of 10 days.

survive higher doses of UV-B (data not shown). Overexpression of both SSB1 and SSB3 enhanced survival to UV-B stress, while that of SSB2, had no effect **(Fig. 5.9B)**. At lower doses of UV-B i.e. 0.75 kJ m^{-2}, SSB1 overexpressing cells exhibited marginally better survival (80.7%) compared to An*ssb3*$^{+}$ (77.4 %), while at higher dose of 1.5 kJ m^{-2}, An*ssb3*$^{+}$ exhibited far better survival (51.3%) as compared to <20% for the other recombinant *Anabaena* strains **(Fig. 5.9B)**.

(c) γ-irradiation stress

Tolerance of the three recombinant *Anabaena* strains (An*ssb*1^{+}, An*ssb*2^{+} and An*ssb*3^{+}) was compared with that of the vector control AnpAM immediately after exposure to γ-irradiation stress (6 kGy of ^{60}Co γ-irradiation; dose rate of 4.6 kGy h^{-1}) and during post irradiation recovery (PIR). The survival measured in terms of CFUs immediately after irradiation indicated about 54% survival for AnpAM, which increased marginally to about 60% upon overexpression of either SSB2 or SSB3, but decreased to ~ 5% upon overexpression of SSB1 **(Fig. 5.10A)**. The lower survival of An*ssb1*$^{+}$ cells was also reflected in their decreased ability to recover. Unlike AnpAM cells which exhibited ~60% recovery on day 3, which increased to >90% on day 7, An*ssb1*$^{+}$ cells exhibited <20% recovery by day 5 which increased to ~35% after 7 days **(Fig. 5.10B)**. Though An*ssb2*$^{+}$ and An*ssb3*$^{+}$ exhibited near equal survival upon exposure to γ-irradiation **(Fig. 5.10A)**, SSB3 overexpression aided the cells in recovering much faster. An*ssb3*$^{+}$ exhibited over 100% recovery from day 1, while recovery of An*ssb2*$^{+}$ cells post-irradiation was similar to that of AnpAM cells **(Fig. 5.10B)**.

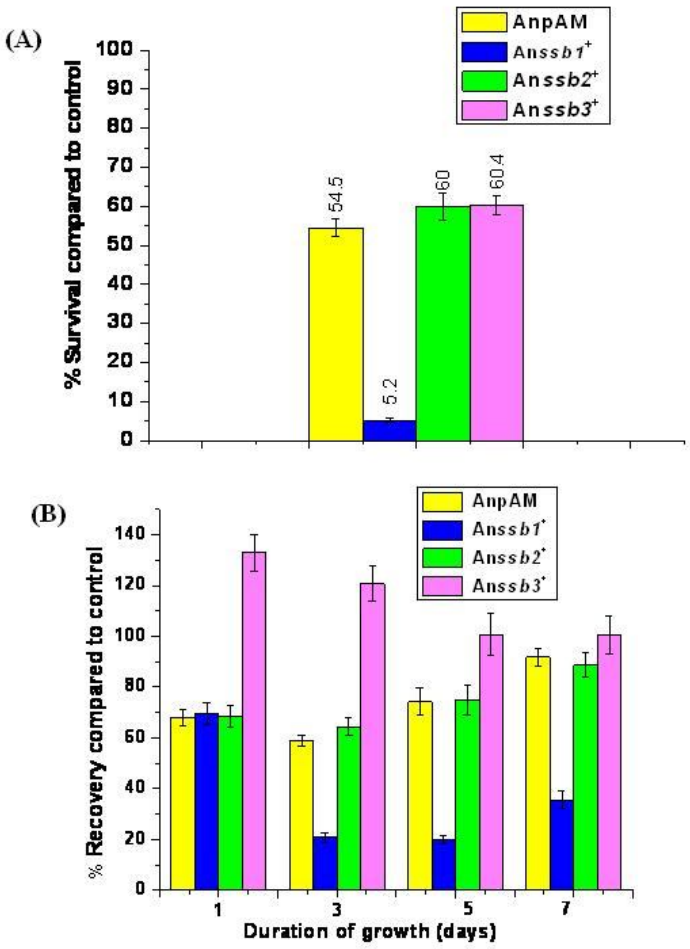

Fig. 5.10 Effect of SSB overexpression on radiation tolerance of *Anabaena*.

Three-day-old recombinant *Anabaena* cultures were concentrated to 10 µg chl*a* density mL^{-1} and exposed to 6 kGy of ^{60}Co γ-irradiation. (A) Survival was measured in terms of colony forming units immediately after irradiation and expressed as percent of the colonies obtained with respective sham-irradiated controls. Other details are as described in legend to Fig. 5.7. (B) The irradiated and control cultures were washed inoculated into fresh BG-11, N^-, $Neo_{12.5}$ medium and allowed to recover under normal growth conditions for 7 days. Growth during post-irradiation recovery was measured in terms of chlorophyll *a* content and expressed as percent of respective control.

(d) Desiccation stress

When subjected to six days of desiccation, the survival of AnpAM, An*ssb1*$^{+}$, An*ssb2*$^{+}$ and An*ssb3*$^{+}$ was about 50%, 54%, 46% and 70% respectively under nitrogen-fixing conditions **(Fig. 5.11A)**, indicating that overexpression of only SSB3 improved the survival of 6 day desiccated *Anabaena* cultures **(Fig. 5.11A)**. The post-desiccation recovery, on the other hand, was affected upon overexpression of SSB1 and SSB2 which resulted in decrease in survival to <20% on day 5 and <15% on day 7 compared to 30% and 55% on day 5 and day 7 for AnpAM cells. Overexpression of SSB3 also enhanced the post-desiccation recovery to ~45% and ~60% on day 5 and day 7 respectively **(Fig. 5.11B)**.

5.3 DISCUSSION

Exposure to chemical agents, such as mitomycin C and radiations, such as UV-B, γ-rays cause damage to DNA, which could be either in the form of DNA adducts or generation of single and double strand breaks (8, 23, 24). Both the kind of damages would interfere with the normal process of DNA replication and recombination, thus affecting cell viability. Thus, these damages need to be repaired. Several DNA repair pathways are known involving different sets of proteins. A common feature of all these pathways is generation of single stranded DNA during the process of repair (192). However, the ssDNA are highly susceptible to the action of nucleases and thus need to be protected, which is offered by SSBs (57). Using the N-terminal OB-fold, SSB protein binds to the ssDNA, and at the same time the C-terminal acidic tail interacts with other

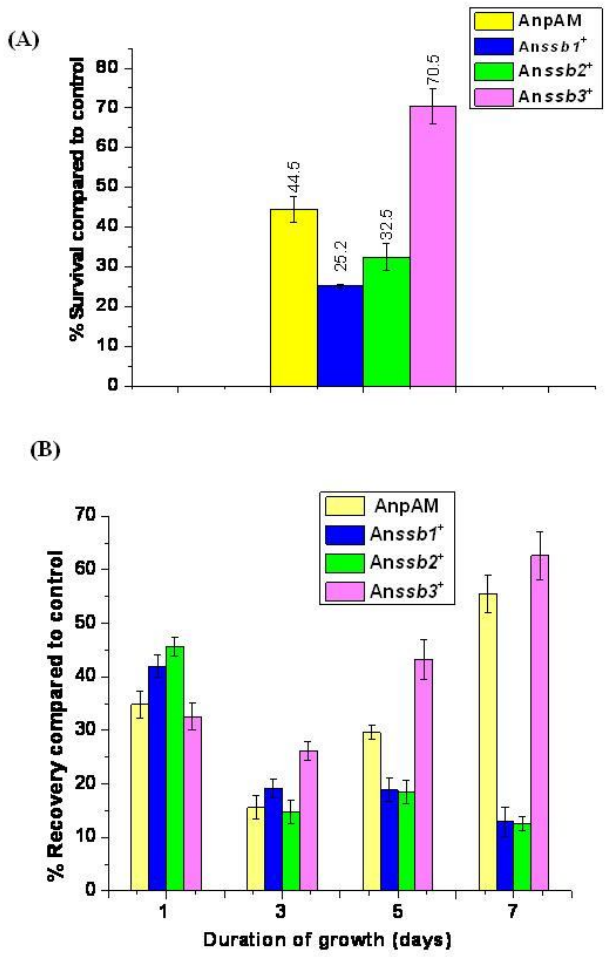

Fig. 5.11 Effect of SSB overexpression on desiccation tolerance of *Anabaena.*

Three-day-old recombinant *Anabaena* cultures were concentrated to 10 µg chl*a* density mL^{-1} and exposed to 6 days of desiccation in a desiccator having fused $CaCl_2$. The corresponding controls were kept in humid chamber for 6 days. (A) Survival was measured in terms of colony forming units immediately after desiccation and expressed as percent of the colonies obtained with respective humid chamber controls. Other details are as described in legend to Fig. 5.7. (B) Growth during post-desiccation recovery was measured in terms of chlorophyll *a* content and expressed as percent of respective control. Other details are as described in legend to Fig. 5.9B.

proteins, thereby helping in bringing together the repair proteins to the site of repair and also induce their activity (6). Thus, it would be envisaged that SSB overexpression would be beneficial to cells, while its deletion mutation would be lethal. In fact, only point mutants of *ssb* have been obtained in bacteria and also these are temperature sensitive and exhibit defect in replication, repair (11). On the other hand, SSB overexpression does not hamper normal cell growth, but all type of repair pathways are not supported possibly due to the non-availability of the required DNA repair proteins. In *E. coli*, SSB overexpression enhances excision repair, but decreases recombinational repair (100).

Anabaena 7120 has three *ssb*-like genes, all of which were found to be essential, since mutation in even one of the *ssb* genes was lethal **(Fig. 5.4)**. This suggested that each of the three *ssb* genes would be playing a specific role, which is not fully compensated by the other two SSBs. To obtain an insight into their possible roles, each of three SSB proteins were individually overexpressed in *Anabaena*. This had no bearing on normal cell growth of *Anabaena* **(Fig. 5.8)**. However, they exhibited differential response to tolerance towards DNA damage inducing stresses. Tolerance of *Anabaena* 7120 exposed to mitomycin C, which induces DNA adduct was enhanced by 20-40% upon overexpression of SSBs **(Fig. 5.9A)**. At a lower dose of mitomycin C (2 μg mL^{-1}), overexpression of SSB3 was more beneficial resulting in over 100% survival as compared to about 83-84% achieved upon overexpressing either SSB1 and SSB2 and 64% for AnpAM cells **(Fig. 5.9A)**. On the other hand, when exposed to a higher dose of mitomycin C (4 μg mL^{-1}), the survival of vector control AnpAM and SSB2 and SSB3 overexpressing recombinant *Anabaena* strains did not change significantly, but An*ssb1*$^{+}$

exhibited very high survival (>100%) **(Fig. 5.8A)**. Of the three SSBs, only SSB3 has an acidic tail and thus could recruit DNA repair proteins, but the survival offered by overexpression of SSB1 was higher. This suggested that the presence of any of the three SSBs in high amounts possibly reduces the severity of damage upon mitomycin C exposure, rather than being involved in repair of DNA. This gains further ground, since exposure to higher concentration of mitomycin C ($\geq$ 6 μg mL^{-1}), or increased duration of exposure at lower doses reduced the survival of AnpAM cells to <10% and was not benefitted by the overexpression of any of the SSB proteins, since exposure to such high or prolonged doses would require the damaged DNA to be repaired for ensuring cell viability.

In case of exposure to UV-B, which also causes formation of DNA adducts i.e. thymine dimers (13), a distinct benefit of SSB overexpression was observed in a UV-dose-dependent manner. At LD_{50} dose of 0.75 kJ m^{-2}, SSB2 overexpression provided no advantage to *Anabaena* in terms of survival, while that of either SSB1 or SSB3 increased survival by 25-30% **(Fig. 5.9B)**. However, at higher doses i.e. 1.5 kJ m^{-2}, which resulted in almost 90% killing, SSB1 overexpression also did not help, but that of SSB3 resulted in over 50% survival. Thus, at higher doses of UV-B, which would require repair of damaged DNA possibly involving UvrABC complex as reported in other bacteria (193), only SSB3 which is theoretically capable of interacting with UvrABC complex offers an advantage when present in large amounts in the cell. The corresponding genes coding for UvrABC are present in *Anabaena* and annotated as *alr3716*, *all1132* and *alr2728*. The involvement of these proteins in excision repair and interaction with SSB3, however, needs to be ascertained.

The ability of SSB3 to aid in enhancing protection against stresses requiring DNA repair is well exemplified by the enhanced protection against radiation and desiccation stresses offered upon overexpression of SSB3, but not SSB1 and SSB2 proteins **(Figs. 5.10 and 5.11)**. In fact overexpression of SSB1, resulted in increase in sensitivity to both these stresses **(Figs. 5.10 and 5.11)**, while that of SSB2 to desiccation stress **(Fig. 5.11)**. Both irradiation and desiccation stresses induce single and double strand breaks resulting in the fragmentation of chromosome (19). Survival against these stresses would require re-stitching of the chromosome in a perfect manner, without any mutation, thus involving several DNA repair proteins in the recombinational repair pathway or NHEJ pathway (194). In archaebacteria, it has been shown that C-terminal deleted RpaC does not offer protection against phleomycin C, which also induces single and double strand breaks (195). Though, the presence of high levels of SSB1 in An*ssb1*$^{+}$ would protect the ssDNA generated in response to γ-irradiation and desiccation stresses against nucleases, the joining of the DNA fragments to form the complete chromosome would not occur due to the absence of C-terminal tail. Also, it may interfere with the functioning of the native SSB protein, resulting in increase in sensitivity of the recombinant *Anabaena* strain. On the other hand, SSB2 which does not exhibit good binding with linear ssDNA, its overexpression does not have a significant effect on post-irradiation recovery of *Anabaena*. Thus, it can be concluded that of the three SSB proteins of *Anabaena*, SSB3, coded by '*all4779*', is the canonical SSB involved in DNA repair process, while the other two SSBs are possibly involved in other essential, yet to be identified processes.

CHAPTER 6

Summary

Single stranded DNA binding (SSB) proteins are present across all genera and perform the function of binding to single stranded (ss) DNA non-specifically and recruitment of proteins involved in DNA repair, recombination and replication. Thus, they form a central part of all DNA-related processes. DNA, in general, is present in a double-stranded (ds) form. However, the above processes require unwinding of DNA, which is carried out by helicases, and also stabilization of the ssDNA generated. Detailed studies on SSB protein of *Escherichia coli* revealed that it plays a crucial role in stabilizing these structures. In general, the SSB protein is structurally divided into three regions. The N-terminal region has an oligonucleotide/oligosaccharide binding (OB)-fold involved in binding to ssDNA, while the C-terminal region is rich in acidic residues, also known as 'acidic tail' and is involved in interaction with other proteins. The middle region is a proline/glycine (P/G)-rich spacer, which ensures proper separation of the positively charged N-terminus from the negatively charged C-terminus. The SSB proteins are usually 178-182 amino acids long, though a few bacterial SSBs have been found to be much shorter, e.g. 113 amino acid long BsSsbB of *Bacillus subtilis* or 140-145 amino acid long SSBs of *Thermatoga* sp.

E. coli SSB exists as a tetramer, with each subunit having a single OB-fold. It binds ssDNA in mainly two binding modes, namely $[SSB]_{35}$, which requires interaction of only two OB-folds with ssDNA, and $[SSB]_{65}$, wherein all the four OB-folds are involved in binding to ssDNA and it results in a wrap-around structure. The SSB proteins of *Deinococcus* and *Thermus* sp. are twice as long as *E. coli* SSB and have two OB-folds per monomer molecule and function as a dimer.

Most bacteria possess only a single *ssb* gene, which is located in the vicinity of DNA repair genes. However, a few bacteria do possess multiple *ssb* genes, such as *B. subtilis*, cyanobacteria. In *B. subtilis*, one of the two SSB proteins, BsSsbA, which is a full length SSB, performs the main role of SSBs, while BsSsbB helps in the development of competence.

In the nitrogen-fixing cyanobacterium, *Anabaena* sp. strain PCC 7120, two genes have been annotated as coding for SSB i.e. *alr0088* and *alr7579*, and have been designated as *ssb1* and *ssb2* in this thesis. Both these genes code for a truncated SSB protein comprising of 119 and 127 amino acids, respectively and comprising of only the N-terminal OB-fold. This is the first report on bacterial SSB proteins lacking both the P/G rich region and the acidic tail. A search in the genome database for a protein likely to be coding for a full length SSB, revealed the presence of an ORF '*all4779*', annotated as a hypothetical protein, which possessed all three regions i.e. the N-terminal OB-fold, a P/G-rich region and a C-terminal acidic tail, typical of bacterial SSB proteins. The protein coded by '*all4779*' was designated as SSB3. Of the three proteins, the overall similarity with bacterial SSB proteins was higher for SSB1 and SSB2 and low for SSB3.

All the three *Anabaena* SSB-like proteins were characterised for their biochemical activity, stress-induced expression and physiological role. The SSB1, SSB2 and SSB3 proteins were individually overexpressed in *E. coli*, by cloning the respective genes in an expression vector, and purified under native conditions. Gel filtration chromatography and glutarladehyde-based cross-linking studies revealed that SSB1 and SSB2 proteins existed either as a monomer or as a dimer under native conditions, while SSB3 formed

low levels of tetramer in the presence of ssDNA. The detection of tetrameric form of SSB3 was possible only upon cross-linking with glutaraldehyde and ssDNA.

The presence of an OB-fold in the three *Anabaena* SSB proteins is suggestive of the ability of these proteins to bind ssDNA. This was tested using two types of substrates i.e. the homopolymeric poly(dT) and heteropolymeric M13 ssDNA or short oligos, and two techniques, i.e. Electrophoretic mobility shift assay (EMSA) and fluorescence quenching. Of the three proteins, SSB2 did not bind poly(dT) effectively, but exhibited maximum binding with M13 ssDNA. On the basis of quenching of the inherent fluorescence of SSB proteins using poly(dT) as a substrate, the binding affinity was found to be maximum for SSB3 and was equivalent to that exhibited by *E. coli* SSB (EcoSSB). The binding affinity of SSB1 was 10-fold lower than that of SSB3. In the absence of acidic tail, the binding affinity of EcoSSB is known to be enhanced by about 10-fold, but the reverse was found to be true for *Anabaena* SSB proteins, and this could be due to the lack of P/G-rich region in addition to the acidic tail in case of SSB1. The SSB2 protein interacted with heteropolymeric ssDNA only. With short oligos, the interaction was observed at high protein to ssDNA ratio, while both SSB1 and SSB3 proteins interacted with short oligos at much lower concentrations of the respective protein. The SSB2 protein, on the other hand, bound only native M13 ssDNA and not heat denatured M13 ssDNA or poly (dT), suggesting that it might be recognizing secondary structures. The affinity of binding of the three SSB proteins to ssDNA assessed by EMSA was in agreement with that calculated on the basis of fluorescence quenching technique. The occlusion site, i.e. the length of ssDNA bound by SSB, was determined on the basis of quenching of fluorescence. Based on this, the SSB1 protein exhibited a single

binding mode of 54-55 bases, while SSB3 exhibited two binding modes, corresponding to 35 bases at low NaCl and ~66 bases at high NaCl. Occlusion site of ~66 bases would require interaction of 4 OB-folds of the SSB protein with the ssDNA i.e. the tetrameric form of the SSB protein. Low levels of the tetrameric form of SSB3 could account for the lower quenching of fluorescence of SSB3 by poly(dT) at high NaCl concentration. Based on the binding affinity data and NaCl concentration-dependent two binding modes of SSB3, in all likelihood, the SSB3 protein of *Anabaena* is the typical bacterial SSB equivalent to EcoSSB and BsSsbA. The low levels of similarity and identity exhibited by SSB3 in comparison with other bacterial SSB proteins could be responsible for it being annotated as a 'hypothetical protein' instead of an SSB.

The presence of multiple SSBs could either indicate specialised function for each of the SSB protein or that only one of them is the true SSB, while the others could have arisen due to gene duplication as has been reported in some bacteria. This was evaluated by assessing stress-induced expression of the three *ssb* genes in *Anabaena* and their role in contributing towards development of tolerance towards DNA-damage inducing stresses. Based on gene organization, bacterial *ssb* genes have been classified under four groups, with Group I and II having the *rpsF-ssb-rpsR* organization and differing only in whether the organism has a single *ssb* gene or multiple *ssb* genes, while Group II refers to those where the *ssb* gene is divergently located to *uvrA*. Those which do not fall in any of these groups have been classified into Group IV. The three *ssb* genes were located in distinct regions of *Anabaena* chromosome and none of the *ssb* genes were adjacent to a DNA repair gene, thus validating the grouping of *Anabaena ssb* genes in Group IV of bacterial *ssb* genes. Bioinformatic analysis revealed the presence of *ssb1* as a

monocistronic gene, *ssb2* either as a single entity or as the first gene of a polycistronic operon, having a maximum of four genes. The genes flanking *ssb3* on either side were transcribed in the same direction as *ssb3*, suggesting that it could be the central gene of a tricistroinc operon. Based on Reverse Transcriptase-PCR (RT-PCR) analysis, it was concluded that *ssb3* is part of a bicistronic operon and transcribed from its own promoter. Detection of transcripts as well as proteins corresponding to *ssb1, ssb2* and *ssb3* under normal growth conditions suggested that all the three *ssb* genes are expressed in *Anabaena* under nitrogen-fixing conditions.

The effect of exposure of *Anabaena* 7120 to different DNA-damage inducing stresses, such as mitomycin C, UV-B radiation, γ-radiation and desiccation, on the expression of the three *ssb* genes was assessed at the promoter as well as protein level. To identify an active promoter for each of the *ssb* genes, a 350-400 bp region upstream of the *ssb* genes was cloned in a promoterless vector, pAM1956 and introduced into *Anabaena* by tri-parental conjugation. Promoter activity was assessed in terms of fluorescence exhibited by Green Fluorescent Protein (GFP), coded by *gfpmutII* used as a reporter gene. Of the two *ssb* genes coding for truncated SSBs, promoter of *ssb1* exhibited higher activity than that of *ssb2*. Both the promoters have a LexA-binding box, which differed in the orientation and length of the palindromic arms, but both interacted with native LexA protein from *Anabaena* 7120. The 350 bp region upstream of *ssb3*, corresponding to the putative promoter region did not exhibit any activity in terms of GFP expression, in *Anabaena*, but was active in *E. coli*. The region 150 bp to 350 bp upstream of the *ssb3* promoter was AT-rich and deletion of this region resulted in an active promoter in *Anabaena*. Thus, *ssb3* transcription is negatively regulated by an AT-

rich region probably requiring a protein specific to *Anabaena*. Though SSB3 seems to be closer to other bacterial SSBs in terms of biochemical activity, the regulation of the truncated *ssb*s of *Anabaena* seems to be following the known typical regulation of bacterial *ssb*s by LexA.

All three *ssb* genes of *Anabaena* were found to be essential indicating specific roles for these proteins, possibly dependent on the growth conditions or in response to various abiotic stresses. *Anabaena* 7120 cells when exposed to mitomycin C or UV-B radiation, both of which are known to induce formation of DNA adducts, did not significantly affect the expression of the *ssb* genes, except that of SSB3 which increased upon exposure to increasing doses of UV-B. However, constitutive overexpression of the SSB proteins was found to be beneficial to *Anabaena* when exposed to these stresses. Among the three SSBs, SSB1 overexpression enhanced tolerance to mitomycin C and low doses of UV-B, SSB2 to low doses of mitomycin C and SSB3 to both low and high doses of the two stresses. The higher contribution of SSB1 than SSB2 to tolerance against these stresses could be due to the higher affinity of SSB1 to short ssDNA compared to SSB2, and thus would be more effective in protecting the short ssDNA regions generated upon exposure to these stresses. On the other hand, SSB3, which has an acidic tail, suggesting ability to interact with proteins, is able to not only protect ssDNA regions generated at low doses of these stresses, but also possibly aid in repair of these damaged region especially at higher doses. This is best exemplified when the cells were exposed to either γ-radiation or desiccation stresses that cause formation of single and double stranded breaks.

Expression of SSB proteins in *Anabaena* is not significantly affected in response to γ-radiation, but that of SSB2 and SSB3 increases during post-irradiation recovery, a sustained increase observed only in case of SSB3. Desiccation stress, on the other hand, does not affect the expression of SSB1, but increases that of SSB2, while SSB3 expression decreased. Constitutive overexpression of SSB1 decreased both survival and tolerance to γ-radiation and desiccation stresses, while that of SSB2 decreased that to desiccation stress, but did not have any effect on either survival or tolerance to γ-radiation. The large regions of ssDNA generated upon exposure to these stresses would be protected from nucleases upon overexpression of SSB1, but since, SSB1 does not have an acidic tail, recruitment of DNA repair proteins to ensure stitching back of the chromosome would not take place. At the same time it may interfere with the functioning of SSB3, which is enhanced during post-irradiation recovery, resulting in increased sensitivity upon overexpression of SSB1. The low binding ability of SSB2 to ssDNA is possibly responsible for it not having any major effect on the tolerance of this organism to these stresses. SSB3, which is capable of binding to ssDNA and probably interact with DNA repair proteins on account of the acidic tail, enhances tolerance to both these stresses, when constitutively expressed in *Anabaena*. All these results point towards SSB3 (*all4779*) performing the role of typical bacterial SSB protein, while the SSB1 and SSB2 could have other essential, yet to be identified roles. Based on literature on proteins having OB-fold and bearing similarity to SSB proteins, the possible roles could either be (i) in initiating formation of primasome complex during replication initiation, a function performed by PriB, a protein thought to have evolved from SSB, or (ii) in aiding

developing competence in *Anabaena*, by protecting the incoming ssDNA from nuclease attack, as has been postulated for BsSsbB.

References

1. Alberts B, Frey L. **1970.** Bacteriophage T4 gene 32: A structural protein in the replication and recombination of DNA. **Nature**. 227: 1313-1318.
2. Sigal N, Delius H, Kornberg T, Gefter ML, 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: 3537-3541.
3. Weiner JH, Bertsch LL, Kornberg A. **1975.** The deoxyribonucleic acid unwinding protein of Escherichia coli. Properties and functions in replication. **J. Biol. Chem.** 250:1972-1980.
4. Alberts B, Sternglanz R. **1977.** Recent excitement in the DNA replication problem. **Nature**. 269: 655-661.
5. Chase JW, Williams KR. **1986.** Single-stranded DNA binding proteins required for DNA replication. **Annu. Rev. Biochem.** 55: 103-136.
6. Shereda RD, Kozlov AG, Lohman TM, Cox MM, Keck JL. **2008.** SSB as an organizer/mobilizer of genome maintenance complexes. **Crit. Rev. Biochem. Mol. Biol.** 43:289-318.
7. Marceau AH. **2012.** Functions of single-strand DNA-binding proteins in DNA replication, recombination, and repair. **Methods Mol. Biol.** 922: 1-21.
8. Bargonetti J, Champeil E, Tomasz M. **2010.** Differential toxicity of DNA adducts of mitomycin C. **J. Nucleic Acids.** pii: 698960. doi:10.4061/**2010**/698960.
9. Suzuki H, Kilgore WW. **1967.** Effects of mitomycin C on macromolecular synthesis in *Escherichia coli*. **J. Bacteriol.** 93: 675-682.
10. Lusetti SL, Wood EA, Fleming CD, Modica MJ, Korth J, Abbott L, Dwyer DW, Roca AI, Inman RB, Cox MM. **2003.** C-terminal deletions of the *Escherichia coli* RecA

protein. Characterization of *in vivo* and *in vitro* effects. **J. Biol. Chem.** 278: 16372-16380.

11. Meyer RR, Laine PS. **1990.** The single-stranded DNA-binding protein of *Escherichia coli*. **Microbiol Rev.** 54: 342-380.
12. 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: 2706-2711.
13. Sinha RP, Häder DP. **2002**. UV-induced DNA damage and repair: a review. **Photochem. Photobiol. Sci.** 1: 225-236.
14. He YY, Häder D. **2002**. Reactive oxygen species and UV-B: effect on cyanobacteria. **Photochem. Photobiol. Sci.** 1: 729-736.
15. Wilkins RJ. **1973.** Does *Escherichia coli* possess a DNA excision repair system for gamma-ray damage? **Nat. New Biol.** 244: 269-271
16. Hariharan PV, Cerutti PA. **1974**. Excision of gamma-ray damaged thymine by *E. coli* extracts is due to the 5' leads to 3' exonuclease associated with DNA polymerase I. **Biochem. Biophys. Res. Commun.** 61: 971-976.
17. Bonura T, Smith KC, Kaplan HS. **1975**. Enzymatic induction of DNA double-strand breaks in gamma-irradiated *Escherichia coli* K-12. **Proc. Natl. Acad. Sci. U S A.** 72: 4265-4269.
18. Bresler SE, Noskin LA, Suslov AV. **1984**. Induction by gamma irradiation of double-strand breaks of *Escherichia coli* chromosomes and their role in cell lethality. **Biophys. J.** 45:749-754.

19. Battista JR. **1997.** Against all odds: the survival strategies of *Deinococcus radiodurans.* **Annu. Rev. Microbiol.** 51: 203-224.

20. Sommers CH. **2003.** Irradiation of minimally processed meats; in microbial safety of minimally processed foods. Pp 301-318. (eds) J S Novak, G M Sapers and V K Juneja (Florida: CRC Press, Inc.)

21. Joshi B, Schmid R, Altendorf K, Apte SK. **2004.** Protein recycling is a major component of post-irradiation recovery in *Deinococcus radiodurans* strain R1. **Biochem. Biophys. Res. Commun.** 320:1112-1117.

22. Slade D, Radman M. **2011.** Oxidative stress resistance in *Deinococcus radiodurans.* **Microbiol. Mol. Biol. Rev.** 75: 133-191.

23. Bryne RT, Chen SH, Wood EA, Cabot EL, Cox MM. **2014.** *Escherichia coli* genes and pathways involved in surviving extreme exposure to ionizing radiation. **J. Bacteriol.** 196: 3534-3545.

24. Bryne RT, Klingele AJ et al. **2014.** Evolution of extreme radioresistance to ionizing radiation *via* genetic adaptation of DNA repair. **eLife**; 3:e01322.

25. Mattimore V, Battista JR. **1996**. Radioresistance of *Deinococcus radiodurans*: functions necessary to survive ionizing radiation are also necessary to survive prolonged desiccation. **J. Bacteriol.** 178: 633-637.

26. Potts M. **1994.** Desiccation tolerance of prokaryotes. **Microbiol. Rev.** 58: 755-805.

27. Murzin AG. **1993.** OB (oligonucleotide/oligosaccharide binding)-fold: common structural and functional solution for non-homologous sequences. **EMBO J.** 12:861-867.

28. Arcus V. **2002** . OB-fold domains: a snapshot of the evolution of sequence, structure and function. **Curr. Opin. Struct. Biol.** 12: 794-801.

29. Theobald DL, Mitton-Fry RM, Wuttke DS. **2003.** Nucleic acid recognition by OB-fold proteins. **Annu. Rev. Biophys. Biomol. Struct.** 32:115-133.

30. Flynn RL, Zou L. **2010.** Oligonucleotide/oligosaccharide-binding fold proteins: a growing family of genome guardians. **Crit. Rev. Biochem. Mol. Biol.** 45: 266-275.

31. Ginalski K, Kinch L, Rychlewski L, Grishin NV. **2004.** BOF: a novel family of bacterial OB-fold proteins. **FEBS Lett.** 567: 297-301.

32. Raghunathan S, Kozlov AG, Lohman TM, Waksman G. **2000.** Structure of the DNA binding domain of *E. coli* SSB bound to ssDNA. **Nat. Struct. Biol.** 7: 648-652.

33. Williams KR, Spicer EK, LoPresti MB, Guggenheimer RA, Chase JW. **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: 3346-3355.

34. Dabrowski S, Olszewski M, Piatek R, Kur J. **2002.** Novel thermostable ssDNA-binding proteins from *Thermus thermophilus* and *T. aquaticus*-expression and purification. **Protein Expr. Purif.** 26: 131-138.

35. Eggington JM, Haruta N, Wood EA, Cox MM. **2004.** The single-stranded DNA-binding protein of *Deinococcus radiodurans*. **BMC Microbiol.** Jan 12; 4:2.

36. Wold MS. **1997.** Replication protein A: a heterotrimeric, single-stranded DNA-binding protein required for eukaryotic DNA metabolism. **Annu. Rev. Biochem.** 66: 61-92.

37. Shamoo Y, Friedman AM, Parsons MR, Konigsberg WH, Steitz TA. **1995.** Crystal structure of a replication fork single-stranded DNA binding protein (T4 gp32) complexed to DNA. **Nature.** 376: 362-366.

38. Haseltine CA, Kowalczykowski SC. **2002.** A distinctive single-stranded DNA-binding protein from the Archeon *Solfolobous solfataricus.* **Mol. Microbiol.** 43: 1505-1515.

39. Lohman TM, Ferrari ME. **1994.** *Escherichia coli* single-stranded DNA-binding protein: multiple DNA-binding modes and cooperativities. **Annu. Rev. Biochem.** 63: 527-570.

40. Bujalowski W, Lohman TM. **1986.** *Escherichia coli* single-strand binding protein forms multiple, distinct complexes with single-stranded DNA. **Biochem.** 25: 7799-7802.

41. Lohman TM, Overman LB. **1985.** Two binding modes in *Escherichia coli* single strand binding protein-single stranded DNA complexes. Modulation by NaCl concentration. **J. Biol. Chem.** 260: 3594-3603.

42. Lohman TM, Overman LB, Datta S. **1986.** Salt-dependent changes in the DNA binding co-operativity of *Escherichia coli* single strand binding protein. **J. Mol. Biol.** 187: 603-615.

43. 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: 5803-5807.

44. Lu D, Keck JL. **2008**. Structural basis of *E. coli* single-stranded DNA-binding protein stimulation of Exonuclease I. **Proc Natl Acad Sci U S A.** 105: 9169-9174.

45. Lu D, Windsor MA, Gellman SH, Keck JL. **2009.** Peptide inhibitors identify roles for SSB C-terminal residues in SSB/exonuclease I complex formation. **Biochem.** 48: 6764-6771.

46. Bork JM, Cox MM, Inman RB. **2001.** The RecOR proteins modulate RecA protein function at 5' ends of single-stranded DNA. **EMBO J.** 20: 7313-7322.

47. Kozlov AG, Lohman TM. **2002.** Kinetic mechanism of direct transfer of *Escherichia coli* SSB tetramers between single-stranded DNA molecules. **Biochem.** 41: 11611-11627.

48. Umezu K, Kolodner RD. **1994.** Protein interactions in genetic recombination in *Escherichia coli*. Interactions involving RecO and RecR overcome the inhibition of RecA by single-stranded DNA-binding protein. **J Biol Chem.** 269: 30005-30013.

49. Krause M, Messer W. **1999.** DnaA proteins of *Escherichia coli* and *Bacillus subtilis*: coordinate actions with single-stranded DNA-binding protein and interspecies inhibition during open complex formation at the replication origins. **Gene.** 228: 123-132.

50. Glover BP, McHenry CS. **1998** The chi psi subunits of DNA polymerase III holoenzyme bind to single-stranded DNA-binding protein (SSB) and facilitate replication of an SSB-coated template. **J Biol Chem.** 273: 23476-23484.

51. Kelman Z, Yuzhakov A, Andjelkovic J, O'Donnell M. **1998.** Devoted to the lagging strand-the subunit of DNA polymerase III holoenzyme contacts SSB to promote processive elongation and sliding clamp assembly. **EMBO J.** 17: 2436-2449.

52. Yuzhakov A, Kelman Z, Hurwitz J, O'Donnell M. **1999.** Multiple competition reactions for RPA order the assembly of the DNA polymerase delta holoenzyme. **EMBO J.** 18: 6189-6199.

53. Marceau AH, Bahng S, Massoni SC, George NP, Sandler SJ, Marians KJ, Keck JL. **2011.** Structure of the SSB-DNA polymerase III interface and its role in DNA replication. **EMBO J.** 30: 4236-4247.

54. Naue N, Fedorov R, Pich A, Manstein DJ, Curth U. **2011.** Site-directed mutagenesis of the χ subunit of DNA polymerase III and single-stranded DNA-binding protein of *E. coli* reveals key residues for their interaction. **Nucleic Acids Res.** 39: 1398-1407.

55. 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. **Biol Chem.** 381: 183-192.

56. Costes A, Lecointe F,McGovern S, Quevillon-Cheruel S, Polard P. **2010.** The C-terminal domain of the bacterial SSB protein acts as a DNA maintenance hub at active chromosome replication forks. **PLoS Genet.** 6(12):e1001238. doi:10.1371/journal.pgen.1001238

57. Pestryakov PE, Lavrik OI. **2008**. Mechanisms of single-stranded DNA-binding protein functioning in cellular DNA metabolism. **Biochemistry (Mosc).** 73: 1388-1404

58. Lecointe F, Sérèna C, Velten M, Costes A, McGovernS, MeileJC, Errington J, Ehrlich SD, Noirot P, Polard P. **2007**. Anticipating chromosomal replication fork arrest: SSB targets repair DNA helicases to active forks. **EMBO J.** 26: 4239-4251.

59. Woodman IL, Brammer K, Bolt EL. **2011**. Physical interaction between archaeal DNA repair helicase Hel308 and Replication Protein A (RPA). **DNA Repair (Amst).** 10: 306-313.

60. Komori K, Ishino Y. **2001.** Replication protein A in *Pyrococcus furiosus* is involved in homologous DNA recombination. **J Biol Chem.** 276: 25654-25660.

61. Shereda RD, Bernstein DA, Keck JL. **2007.** A central role for SSB in *Escherichia coli* RecQ DNA helicase function. **J Biol Chem.** 282: 19247-19258.

62. Han ES, Cooper DL, Persky NS, Sutera VA Jr, Whitaker RD, Montello ML, Lovett ST. **2006.** RecJ exonuclease: substrates, products and interaction with SSB. **Nucleic Acids Res.** 34: 1084-1091.

63. Sharma R, Rao DN. **2009.** Orchestration of *Haemophilus influenzae* RecJ exonuclease by interaction with single-stranded DNA-binding protein. **J Mol Biol.** 385: 1375-1396.

64. Tseng YC, Hung JL, Wang TC. **1994.** Involvement of RecF pathway recombination genes in post-replication repair in UV-irradiated *Escherichia coli* cells. **Mutat Res.** 315:1-9.

65. Morimatsu K, Wu Y, Kowalczykowski SC. **2012.** RecFOR proteins target RecA protein to a DNA gap with either DNA or RNA at the 5' terminus: implication for repair of stalled replication forks. **J Biol Chem.** 287: 35621-35630.

66. Ryzhikov M, Koroleva O, Postnov D, Tran A, Korolev S. **2011.** Mechanism of RecO recruitment to DNA by single-stranded DNA binding protein. **Nucleic Acids Res.** 39: 6305-6314.

67. Inoue J, Honda M, Ikawa S, Shibata T, Mikawa T. **2008.** The process of displacing the single-stranded DNA-binding protein from single-stranded DNA by RecO and RecR proteins. **Nucleic Acids Res.** 36: 94-109.

68. Brody RS, Doherty KG, Zimmerman PD. **1986.** Processivity and kinetics of the reaction of exonuclease I from *Escherichia coli* with polydeoxyribonucleotides. **J Biol Chem.** 261: 7136-7143.

69. Phillips GJ, Prasher DC, Kushner SR. **1988.** Physical and biochemical characterization of cloned *sbcB* and *xonA* mutations from *Escherichia coli* K-12. **J Bacteriol.** 170: 2089-2094.

70. Wang TC, Smith KC. **1985.** Mechanism of *sbcB*-suppression of the *recAB*-deficiency in post replication repairin UV-irradiated *Escherichia coli* K12. **Mol. Gen. Genet.** 201: 186-191.

71. Lahue RS, Au KG, Modrich P. **1989.** DNA mismatch correction in a defined system. **Science.** 245: 160-164.

72. Sandigursky M, Franklin WA. **1992.** DNA deoxyribophosphodiesterase of *Escherichia coli* is associated with exonuclease I. **Nucleic Acids Res.** 20: 4699-4703.

73. Sandigursky M, Mendez F, Bases RE, Matsumoto T, Franklin WA. **1996.** Protein-protein interactions between the *Escherichia coli* single-stranded DNA-binding protein and exonuclease I. **Radiat Res.** 145: 619-623.

74. Dianov G, Sedgwick B, Daly G, Olsson M, Lovett S, Lindahl T. **1994.** Release of 5'-terminal deoxyribose-phosphate residues from incised abasic sites in DNA by the *Escherichia coli* RecJ protein. **Nucleic Acids Res.** 22: 993-998.

75. Purnapatre K, Handa P, Venkatesh J, Varshney U. **1999.** Differential effects of single-stranded DNA binding proteins (SSBs) on uracil DNA glycosylases (UDGs) from *Escherichia coli* and mycobacteria. **Nucleic Acids Res.** 27: 3487-3492.

76. D'souza DI, Harrison L. **2003**. Repair of clustered uracil DNA damages in *Escherichia coli*. **Nucleic Acids Res.** 31: 4573-4581.

77. Kumar NV, Varshney U. **1997.** Contrasting effects of single stranded DNA binding protein on the activity of uracil DNA glycosylase from *Escherichia coli* towards different DNA substrates. **Nucleic Acids Res.** 25: 2336-2343.

78. Iwasaki H, Nakata A, Walker GC, Shinagawa H. **1990.** The *Escherichia coli polB* gene, which encodes DNA polymerase II, is regulated by the SOS system. **J Bacteriol.** 172: 6268-6273.

79. Pages V, Janel-Bintz R, Fuchs RP. **2005.** Pol III proofreading activity prevents lesion bypass as evidenced by its molecular signature within *E. coli* cells. **J Mol Biol.** 352: 501-509.

80. Sedliakova M, Slezarikova V, Masek F, Vizvaryova M, Pirsel M. **2001**. Role of DNA polymerase II in the tolerance of thymine dimers remaining unexcised in UV-irradiated Escherichia coli exposed to pre-UV nutritional stress. **J Photochem Photobiol B.** 65: 145-150.

81. Molineux IJ, Gefter ML **1974.** Properties of the *Escherichia coli* in DNA binding (unwinding) protein: interaction with DNA polymerase and DNA. **Proc Natl Acad Sci U S A.** 71: 3858-3862.

82. Dalrymple BP, Kongsuwan K, Wijffels G, Dixon NE, Jennings PA. **2001.** A universal protein-protein interaction motif in the eubacterial DNA replication and repair systems. **Proc Natl Acad Sci U S A.** 98: 11627-11632.

83. Becherel OJ, Fuchs RP, Wagner J. **2002.** Pivotal role of the beta-clamp in translesion DNA synthesis and mutagenesis in *E. coli* cells. **DNA Repair (Amst).** 1: 703-708.

84. Goodman MF. **2000**. Coping with replication 'train wrecks' in *Escherichia coli* using Pol V, Pol II and RecA proteins. **Trends Biochem Sci.** 25: 189-195.

85. Tang M, Shen X, Frank EG, O'Donnell M, Woodgate R, Goodman MF. **1999.** UmuD'(2)C is an error-prone DNA polymerase, *Escherichia coli* pol V. **Proc Natl Acad Sci U S A.** 96: 8919-8924.

86. Arad G, Hendel A, Urbanke C, Curth U, Livneh Z. **2008**. Single-stranded DNA-binding protein recruits DNA polymerase V to primer termini on RecA-coated DNA. **J Biol Chem.** 283: 8274-8282.

87. Glucksmann-Kuis MA, Dai X, Markiewicz P, Rothman-Denes LB. **1996.** *E. coli* SSB activates N4 virion RNA polymerase promoters by stabilizing a DNA hairpin required for promoter recognition. **Cell.** 84: 147-154

88. Richard DJ, Bell SD, White MF. **2004.** Physical and functional interaction of the archaeal single-stranded DNA-binding protein SSB with RNA polymerase. **Nucleic Acids Res.** 32: 1065-1074.

89. Mijakovic I, Petranovic D, Macek B, Cepo T, Mann M, Davies J, Jensen PR, Vujaklija D. **2006.** Bacterial single-stranded DNA-binding proteins are phosphorylated on tyrosine. **Nucleic Acids Res.** 34: 1588-1596.

90. Vujaklija D, Macek B. **2012.** Detecting posttranslational modifications of bacterial SSB proteins. **Methods Mol Biol.** 922: 205-218.

91. Hass CS, Chen R, Wold MS. **2012.** Detection of posttranslational modifications of replication protein A. **Methods Mol Biol.** 922: 193-204.

92. Nuss JE, Patrick SM, Oakley GG, Alter GM, Robison JG, Dixon K, Turchi JJ. **2005.** DNA damage induced hyperphosphorylation of replication protein A. 1. Identification of novel sites of phosphorylation in response to DNA damage. **Biochemistry.** 44: 8428-8437.

93. Richard DJ, Bolderson E, Khanna KK. **2009.** Multiple human single-stranded DNA binding proteins function in genome maintenance: structural, biochemical and functional analysis. **Crit Rev Biochem Mol Biol.** 44: 98-116.

94. Dou H, Huang C, Singh M, Carpenter PB, Yeh ET. **2010**. Regulation of DNA repair through deSUMOylation and SUMOylation of replication protein A complex. **Mol Cell.** 39: 333-345.

95. Meyer RR, Glassberg J, Kornberg A. **1979.** An *Escherichia coli* mutant defective in single-stranded binding protein is defective in DNA replication. **Proc Natl Acad Sci U S A.** 76: 1702-1705.

96. Chase JW, L'Italien JJ, Murphy JB, Spicer EK, Williams KR. **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: 805-814.

97. Wang TC, Smith KC. **1982.** Effects of the *ssb*-1 and *ssb*-113 mutations on survival and DNA repair in UV-irradiated delta *uvr*B strains of *Escherichia coli* K-12. **J Bacteriol.** 151: 186-192.

98. Lieberman HB, Witkin EM. **1981.** Variable expression of the *ssb*-1 allele in different strains of *Escherichia coli* K12 and B: differential suppression of its effects on DNA replication, DNA repair and ultraviolet mutagenesis. **Mol Gen Genet.** 183: 348-355.

99. Williams KR, Murphy JB, Chase JW. **1984.** Characterization of the structural and functional defect in the *Escherichia coli* single-stranded DNA binding protein encoded by the *ssb*-1 mutant gene. Expression of the *ssb*-1 gene under lambda pL regulation. **J Biol Chem.** 259: 11804-11811.

100. Moreau PL. **1987.** Effects of overproduction of single-stranded DNA-binding protein on RecA protein-dependent processes in *Escherichia coli*. **J Mol Biol.** 194: 621-634.

101. Moreau PL. **1988.** Overproduction of single-stranded-DNA-binding protein specifically inhibits recombination of UV-irradiated bacteriophage DNA in *Escherichia coli*. **J Bacteriol.** 170: 2493-2500.

102. Dri AM, Moreau PL. **1991.** Properties of RecA441 protein reveal a possible role for RecF and SSB proteins in *Escherichia coli*. **Mol Gen Genet.** 227: 488-492.

103. Salaj-Smic E, Lers N, Trgovcević Z. **1988.** Overproduction of single-stranded DNA-binding protein increases UV-induced mutagenesis in *Escherichia coli*. **Mutat Res.** 208: 179-182.

104. Lers N, Salaj-Smic E, Trgovcević Z. **1989.** Overproduction of SSB protein enhances the capacity for photorepair in *Escherichia coli recA* cells. **Photochem Photobiol.** 49: 225-227.

105. Lindner C, Nijland R, van Hartskamp M, Bron S, Hamoen LW, Kuipers OP. **2004**. Differential expression of two paralogous genes of *Bacillus subtilis* encoding single-stranded DNA binding protein. **J Bacteriol.** 186: 1097-1105.

106. Yadav T, Carrasco B, Myers AR, George NP, Keck JL, Alonso JC. **2012.** Genetic recombination in *Bacillus subtilis*: a division of labor between two single-strand DNA-binding proteins. **Nucleic Acids Res.** 40: 5546-5559.

107. Kidane D, Ayora S, Sweasy JB, Graumann PL, Alonso JC. **2012.** The cell pole: the site of cross talk between the DNA uptake and genetic recombination machinery. **Crit Rev Biochem Mol Biol.** 47: 531-555.

108. Bernstein DA, Eggington JM, Killoran MP, Misic AM, Cox MM, Keck JL. Crystal structure of the *Deinococcus radiodurans* single-stranded DNA-binding protein suggests a mechanism for coping with DNA damage. **2004. Proc Natl Acad Sci U S A.** 101: 8575-8580.

109. Norais CA, Chitteni-Pattu S, Wood EA, Inman RB, Cox MM. **2009.** DdrB protein, an alternative *Deinococcus radiodurans* SSB induced by ionizing radiation. **J Biol Chem.** 284: 21402-21411.

110. Sugiman-Marangos S, Junop MS. **2010**. The structure of DdrB from *Deinococcus*: a new fold for single-stranded DNA binding proteins. **Nucleic Acids Res.** 38: 3432-3440.

111. Bouthier de la Tour C, Boisnard S, Norais C, Toueille M, Bentchikou E, Vannier F, Cox MM, Sommer S, Servant P. **2011**. The *deinococcal* DdrB protein is involved in an early step of DNA double strand break repair and in plasmid transformation through its single-strand annealing activity. **DNA Repair (Amst).** 10: 1223-1231.

112. Lockhart JS, DeVeaux LC. **2013.** The essential role of the Deinococcus radiodurans ssb gene in cell survival and radiation tolerance **PLoS One.** 8(8):e71651. doi: 10.1371/journal.pone.0071651.

113. Yang C, Curth U, Urbanke C, Kang C. **1997**. Crystal structure of human mitochondrial single-stranded DNA binding protein at 2.4 Å resolution. **Nat Struct Biol.** 4: 153-157.

114. Bochkarev A, Pfuetzner RA, Edwards AM, Frappier L. **1997.** Structure of the single-stranded-DNA-binding domain of replication protein A bound to DNA. **Nature.** 385: 176-181.

115. Oliveira MT, Kaguni LS. **2010.** Functional roles of the N- and C-terminal regions of the human mitochondrial single-stranded DNA-binding protein. **PLoS One.** 5(10):e15379. doi: 10.1371/journal.pone.0015379.

116. Brandsma JA, Bosch D, de Ruÿter M, van de Putte P. **1985.** Analysis of the regulatory region of the *ssb* gene of *Escherichia coli*. **Nucleic Acids Res.** 13: 5095-5109.

117. Brandsma JA, Bosch D, Backendorf C, van de Putte P. **1983.** A common regulatory region shared by divergently transcribed genes of the *Escherichia coli* SOS system. **Nature.** 305: 243-245.

118. Perrino FW, Rein DC, Bobst AM, Meyer RR. **1987.** The relative rate of synthesis and levels of single-stranded DNA binding protein during induction of SOS repair in *Escherichia coli*. **Mol Gen Genet.** 209: 612-614.

119. Ujaoney AK, Potnis AA, Kane P, Mukhopadhyaya R, Apte SK. **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: 5637-5644.

120. Shimamoto N, Ikushima N, Utiyama H, Tachibana H, Horie K. **1987.** Specific and cooperative binding of *E. coli* single-stranded DNA binding protein to mRNA. **Nucleic Acids Res.** 15: 5241-5250.

121. Brock TD. **1973.** Lower pH limit for the existence of blue-green algae: evolutionary and ecological implications. **Science.** 179: 480-483.

122. Fay P. **1992.** Oxygen relations of nitrogen fixation in cyanobacteria. **Microbiol Rev.** 56: 340-373.

123. Schopf JW. **1975.** Precambrian Paleobiology: Problems and Perspectives. **Ann. Rev. Earth Planetary Sci.** 3: 213-249.

124. Stanier RY, Cohen-Bazire G. **1977.** Phototrophic prokaryotes: the cyanobacteria. **Annu. Rev. Microbiol.** 31: 225-274.

125. Mandel M. **1969**. New approaches to bacterial taxonomy: perspective and prospects. **Annu. Rev. Microbiol.** 23: 239-274.

126. Los DA, Murata N. **1999.** Responses to cold shock in cyanobacteria. **J. Mol. Microbiol. Biotechnol.** 1: 221-230.

127. Stewart AC, Bendall DS. **1981.** Properties of oxygen-evolving photosystem-II particles from *Phormidium laminosum*, a thermophilic blue--green alga. **Biochem. J.** 194: 877-887.

128. Walsby AE. **1986.** Prochlorophytes: Origin of chloroplasts. **Nature** 320: 212.

129. Adams DG. 2000 The Ecology of Cyanobacteria Their Diversity in Time and Space Whitton, B.A.; Potts, M. (Eds.) 2000, XVIII, **523-561**

130. Stewart WD. 1973 Nitrogen fixation by photosynthetic microorganisms **Annu. Rev. Microbiol.** 27: 283-316.

131. Castenholz RW. **1969**. Thermophilic blue-green algae and the thermal environment. **Bacteriol Rev.** 33: 476-504.

132. Fogg GE, Stewart WDP, Fay P, Walsby AE. **1973.** The Blue-Green Algae (London and NY; Academic Press).

133. Stewart WD. **1980.** Some aspects of structure and function in N_2-fixing cyanobacteria. **Annu Rev Microbiol.** 34:497-536.

134. Weare NM, Benemann JR. **1974.** Nitrogenase activity and photosynthesis in *Plectonema boryanum*. **J Bacteriol.** 119: 258-265.

135. Rabouille S, Van de Waal DB, Matthijs HC, Huisman J. **2014**. Nitrogen fixation and respiratory electron transport in the cyanobacterium cyanothece under different light:dark cycles. **FEMS Microbiol Ecol.** 87:630-638.

136. Wolk CP. **1996.** Heterocyst formation. **Annu Rev Genet.** 30:59-78.

137. Adams DG. **2000.** Heterocyst formation in cyanobacteria. **Curr Opin Microbiol.** 3: 618-624.

138. Stewart WD, Rowell P, Tel-or E. **1975.** Nitrogen fixation and the heterocyst in blue-green algae. **Biochem Soc Trans.** 3: 357-361.

139. Häder DP, Sinha RP. **2005.** Solar ultraviolet radiation-induced DNA damage in aquatic organisms: potential environmental impact. **Mutat Res.** 571: 221-233.

140. Miranda, H. **2011.** Stress response in cyanobacterium *Synechocystis* sp. PCC 6803. Doctoral Thesis, Department of Chemistry, Umeå University, Sweden.

141. Bhagwat AA, Apte SK. **1989.** Comparative analysis of proteins induced by heat shock, salinity, and osmotic stress in the nitrogen-fixing cyanobacterium *Anabaena* sp. strain L-31. **J Bacteriol.** 171: 5187-5189.

142. Apte SK. **2001.** Coping with salinity/water stress: Cyanobacteria show the way. **Proc. Indian Natl. Acad. Sci** (PINSA) B67: 285-310.

143. Janion C. **2008.** Inducible SOS response system of DNA repair and mutagenesis in *Escherichia coli*. **Int J Biol Sci.** 4: 338-344.

144. Sode K, Oozeki M, Asakawa K, Burgess J, Matusunaga T. **1994.** Isolation of a marine cyanophage infecting the unicellular cyanobacterium *Synechococcus* species NKBG042902. **J. Mar. Biotechnol.** 1: 189–192.

145. Tetu SG, Johnson DA, Varkey D, Phillippy K, Stuart RK, Dupont CL, Hassan KA, Palenik B, Paulsen IT. **2013.** Impact of DNA damaging agents on genome-wide transcriptional profiles in two marine *Synechococcus* species. **Front Microbiol.** 4: 232. doi: 10.3389/fmicb.**2013**.00232.

146. Owttrim GW, Coleman JR. **1989.** Regulation of expression and nucleotide sequence of the *Anabaena variabilis recA* gene. **J Bacteriol.** 171: 5713-5719.

147. Häder DP, Kumar HD, Smith RC, Worrest RC. **2007.** Effects of solar UV radiation on aquatic ecosystems and interactions with climate change. **Photochem Photobiol Sci.** 6: 267-285.

148. Hargreaves A, Taiwo FA, Duggan O, Kirk SH, Ahmad SI. **2007.** Near-ultraviolet photolysis of beta-phenylpyruvic acid generates free radicals and results in DNA damage. **J Photochem Photobiol B.** 89: 110-116.

149. Singh SP, Häder DP, Sinha RP. **2010.** Cyanobacteria and ultraviolet radiation (UVR) stress: mitigation strategies. **Ageing Res Rev.** 9: 79-90.

150. Gao K, Yu H, Brown MT. **2007.** Solar PAR and UV radiation affects the physiology and morphology of the cyanobacterium *Anabaena* sp. PCC 7120. **J Photochem Photobiol B.** 89: 117-124.

151. Häder DP, Häder M. **1990.** Effects of UV radiation on motility, photo-orientation and pigmentation in a freshwater Cryptomonas. **J Photochem Photobiol B.** 5: 105-114.

152. Sinha RP, Singh N, Kumar A, Kumar HD, Ha¨der M, Ha¨der D-P. **1996.** Effects of UV irradiation on certain physiological and biochemical processes in cyanobacteria. **J. Photochem. Photobiol. B Biol.** 32: 107–113.

153. Kumar A, Tyagi MB, Jha PN, Srinivas G, Singh A. **2003.**Inactivation of cyanobacterial nitrogenase after exposure to ultraviolet-B radiation. **Curr Microbiol.** 46: 380-384.

154. Bhargava P, Srivastava AK, Urmil S, Rai LC. **2005**. Phytochelatin plays a role in UV-B tolerance in N_2-fixing cyanobacterium *Anabaena doliolum*. **J Plant Physiol.** 162: 1220-1225.

155. Kojima K, Oshita M, Nanjo Y, Kasai K, Tozawa Y, Hayashi H, Nishiyama Y. **2007**.Oxidation of elongation factor G inhibits the synthesis of the D1 protein of photosystem II. **Mol Microbiol.** 65: 936-947.

156. Asato Y. **1971.** Photorecovery of gamma irradiated cultures of bluegreen alga, *Anacystis nidulans*. **Rad. Bot.** 11**:** 313–316

157. Dmitriev AP, Grodzinskiĭ DM. **1973**. Combined effect of chemical compounds and gamma-radiation on cells of the blue-green alga *Anacystis nidulans*. **Mikrobiologiia.** 42: 307-311.

158. Agarwal R, Rane SS, Sainis JK. **2008.** Effects of (60)Co gamma radiation on thylakoid membrane functions in *Anacystis nidulans*. **J Photochem Photobiol B.** 91: 9-19.

159. Kraus MP. **1969.** Resistance of blue-green algae to ^{60}Co gamma radiation. **Radiat. Biol.** 9**:** 481–489.

160. Billi D, Friedmann EI, Hofer KG, Caiola MG, Ocampo-Friedmann R. **2000.** Ionizing-radiation resistance in the desiccation-tolerant cyanobacterium *Chroococcidiopsis*. **Appl Environ Microbiol.** 66: 1489-1492.

161. Singh H, Fernandes T, Apte SK. **2010**. Unusual radioresistance of nitrogen-fixing cultures of *Anabaena* strains. **J Biosci.** 35: 427-434.

162. Singh H, Anurag K, Apte SK. **2013.** High radiation and desiccation tolerance of nitrogen-fixing cultures of the cyanobacterium *Anabaena* sp. strain PCC 7120 emanates from genome/proteome repair capabilities. **Photosynth Res.** 118: 71-81.

163. Simon RD, Lawry NH, McLendon GL. **1980.** Structural characterization of the cyanophycin granule polypeptide of *Anabaena cylindrica* by circular dichroism and Raman spectroscopy. **Biochim Biophys Acta.** 626: 277-281.

164. Daly MJ, Gaidamakova EK, Matrosova VY, Vasilenko A, Zhai M, Venkateswaran A, Hess M, Omelchenko MV, Kostandarithes HM, Makarova KS, Wackett LP, Fredrickson JK, Ghosal D. **2004.** Accumulation of Mn(II) in *Deinococcus radiodurans* facilitates gamma-radiation resistance. **Science.** 306: 1025-1028.

165. Potts M. **1999.** Mechanism of desiccation tolerance in Cyanobacteria. **Europian J. Phycol.** 34: 319-328.

166. Adams DG, Carr NG. **1981.** The developmental biology of heterocyst and akinete formation in cyanobacteria. **Crit Rev Microbiol.** 9: 45-100.

167. Tamaru Y, Takani Y, Yoshida T, Sakamoto T. **2005.** Crucial role of extracellular polysaccharides in desiccation and freezing tolerance in the terrestrial cyanobacterium *Nostoc commune*. **Appl Environ Microbiol.** 71: 7327-7333.

168. Kaneko T, Nakamura Y, Wolk CP, Kuritz T, Sasamoto S, Watanabe A, Iriguchi M, Ishikawa A, Kawashima K, Kimura T, Kishida Y, Kohara M, Matsumoto M, Matsuno A, Muraki A, Nakazaki N, Shimpo S, Sugimoto M, Takazawa M, Yamada M, Yasuda M, Tabata S. **2001.** Complete genomic sequence of the filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120. **DNA Res.** 8: 205-213; 227-253.

169. Wolk CP, Vonshak A, Kehoe P, Elhai J. **1984.** Construction of shuttle vectors capable of conjugative transfer from *Escherichia coli* to nitrogen-fixing filamentous cyanobacteria. **Proc Natl Acad Sci U S A.** 81: 1561-1565.

170. Golden JW, Carrasco CD, Mulligan ME, Schneider GJ, Haselkorn R. **1988.** Deletion of a 55-kilobase-pair DNA element from the chromosome during heterocyst differentiation of *Anabaena* sp. strain PCC 7120. **J Bacteriol.** 170: 5034-5041.

171. CaiYP, Wolk CP. **1990.** Use of a conditionally lethal gene in *Anabaena* sp. strain PCC 7120 to select for double recombinants and to entrap insertion sequences. **J Bacteriol.** 172: 3138-3145.

172. Chaurasia AK, Parasnis A, Apte SK. **2008.** An integrative expression vector for strain improvement and environmental applications of the nitrogen fixing cyanobacterium, *Anabaena* sp. strain PCC7120. **J Microbiol Methods.** 73: 133-141.

173. Rajaram H, Apte SK. **2008.** Nitrogen status and heat-stress-dependent differential expression of the *cpn60* chaperonin gene influences thermotolerance in the cyanobacterium *Anabaena*. **Microbiol.** 154: 317-325.

174. Chaurasia AK, Apte SK. **2009.** Overexpression of the *groESL* operon enhances the heat and salinity stress tolerance of the nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC7120. **Appl Environ Microbiol.** 75: 6008-6012.

175. Raghavan PS, Rajaram H, Apte SK. **2011.** Nitrogen status dependent oxidative stress tolerance conferred by overexpression of MnSOD and FeSOD proteins in *Anabaena* sp. strain PCC7120. **Plant Mol Biol.** 77: 407-417.

176. Castenholz RW. **1988.** Culturing of cyanobacteria. **Methods Enzymol.** 167: 68-93.

177. Mackinney G. **1941.** Absorption of light by chlorophyll solutions. **J Biol Chem.** 140: 315-322.

178. Yoon HS, Golden JW. **1998.** Heterocyst pattern formation controlled by a diffusible peptide. **Science.** 282: 935-938.

179. Rajaram H, Apte SK. **2010.** Differential regulation of *groESL* operon expression in response to heat and light in *Anabaena*. **Arch. Microbiol.** 192: 729-738.

180. Laemmli UK. **1970.** Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature.** 227: 680-685.

181. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. **1951.** Protein measurement with the Folin phenol reagent **J Biol Chem**. 193: 265-275.

182. Dabrowski S, Kur J. **1999.** Cloning, overexpression, and purification of the recombinant His-tagged SSB protein of *Escherichia coli* and use in polymerase chain reaction amplification. **Protein Expr Purif.** 16: 96-102.

183. Molineux IJ, Pauli A, Gefter ML. **1975.** Physical studies of the interaction between the *Escherichia coli* DNA binding protein and nucleic acids. **Nucleic Acids Res.** 2: 1821-1837.

184. Jain S, Zweig M, Peeters E, Siewering K, Hackett KT, Dillard JP, van der Does C. **2012.** Characterization of the single stranded DNA binding protein SsbB encoded in the Gonoccocal Genetic Island. **PLoS One.** 7(4):e35285. doi: 10.1371/journal.pone.0035285.

185. Olszewski M, Grot A, Wojciechowski M, Nowak M, Mickiewicz M, Kur J. **2010.** Characterization of exceptionally thermostable single-stranded DNA-binding proteins from *Thermotoga maritima* and *Thermotoga neapolitana*. **BMC Microbiol.** 10:260. doi: 10.1186/1471-2180-10-260.

186. Kozlov AG, Cox MM, Lohman TM. **2010.** Regulation of single-stranded DNA binding by the C termini of *Escherichia coli* single-stranded DNA binding (SSB) protein. **J. Biol. Chem.** 285: 17246-17252.

187. Belts MJ, Russell RB. **2003.** Amino acid properties and consequences of substitutions. In Bioinformatics for Geneticists. Ed. Barnes MR and Gray IC. John Wiley and sons, pp 289-316.

188. Reckmann B, Grosse F, Urbanke C, Frank R, Blocker H, Krauss G. **1985.** Analysis of secondary structures in M13mp8 (+) single-stranded DNA by the pausing of DNA Polymerase α. **Eur. J. Biochem.** 152: 633-643.

189. Ponomarev VA, Makarova KS, Aravind L, Koonin EV. **2003.** Gene duplication with displacement and rearrangement: origin of the bacterial replication protein PriB from the single-stranded DNA-binding protein, Ssb. **J. Mol. Microbiol. Biotechnol.** 5: 225-229.

190. Yura K, Toh H, Go M. **1999.** Putative mechanism of natural transformation as deduced form genome data. **DNA Res.** 6: 75-82.

191. Nakasugi K, Svenson CJ, Neilan BA. **2006.** The competence gene, *comF*, from *Synechocystis* sp. strain PCC6803 is involved in natural transformation, phototactic motility and piliation. **Microbiol.** 152: 3623-3631.

192. Marceau AH. **2012.** Functions of single-strand DNA-binding proteins in DNA replication, recombination, and repair. **Methods Mol Biol.** 922: 1-21.

193. Van Houten B. **1990.** Nucleotide excision repair in *Escherichia coli*. **Microbiol Rev.** 54: 18-51.

194. Lieber MR **2010.** The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway. **Annu Rev Biochem.** 79: 181-211.

195. Skowyra A, MacNeill SA. **2012**. Identification of essential and non-essential single-stranded DNA-binding proteins in a model archaeal organism. **Nucleic Acids Res.** 40: 1077-1090.

List of Publications

1. Anurag Kirti, Hema Rajaram***** and Shree Kumar Apte (2013) Characterization of two naturally truncated, Ssb-like proteins from the nitrogen-fixing cyanobacterium, *Anabaena* sp. PCC7120. **Photosyn. Res**. 118: 147-154.
2. Anurag Kirti, Hema Rajaram***** and Shree Kumar Apte (2014) The hypothetical protein All4779 and not the annotated 'Alr0088' and 'Alr7579' protein is the major typical single-stranded DNA binding protein of the cyanobacterium, *Anabaena* sp. PCC7120. **PLoS ONE**, 9(4): e93592. doi:10.1371/journal.pone.0093592

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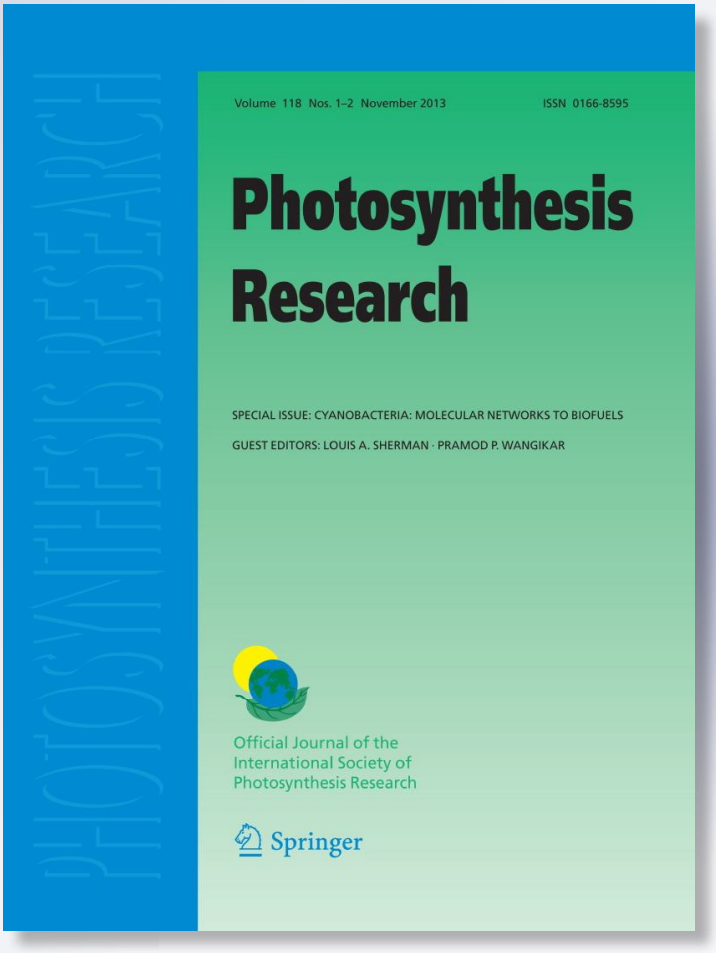

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REGULAR PAPER

Characterization of two naturally truncated, Ssb-like proteins from the nitrogen-fixing cyanobacterium, *Anabaena* sp. PCC7120

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Abstract Single-stranded (ss) DNA-binding (Ssb) proteins are vital for all DNA metabolic processes and are characterized by an N-terminal OB-fold followed by P/G-rich spacer region and a C-terminal tail. In the genome of the heterocystous, nitrogen-fixing cyanobacterium, *Anabaena* sp. strain PCC 7120, two genes *alr0088* and *alr7579* are annotated as *ssb*, but the corresponding proteins have only the N-terminal OB-fold and no P/G-rich region or acidic tail, thereby rendering them unable to interact with genome maintenance proteins. Both the proteins were expressed under normal growth conditions in *Anabaena* PCC7120 and regulated differentially under abiotic stresses which induce DNA damage, indicating that these are functional genes. Constitutive overexpression of Alr0088 in *Anabaena* enhanced the tolerance to DNA-damaging stresses which caused formation of DNA adducts such as UV and MitomycinC, but significantly decreased the tolerance to γ-irradiation, which causes single- and double-stranded DNA breaks. On the other hand, overexpression of Alr7579 had no significant effect on normal growth or stress tolerance of *Anabaena*. Thus, of the two truncated Ssb-like proteins, Alr0088 may be involved in protection of ssDNA from damage, but due to the absence of acidic tail, it may not aid in repair of damaged DNA. These two proteins are present across cyanobacterial genera and unique to them. These initial studies pave the way to the understanding of DNA repair in cyanobacteria, which is not very well documented.

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Introduction

Oligonucleotide/oligosaccharide (OB) folds are present in a wide range of DNA/RNA binding proteins, e.g. anticodon-binding domain of t-RNA synthetase, ss-DNA-binding domains, super antigen binding domains, some bacterial toxins, DNA ligase, Primase, etc. (Theobald et al. 2003). The OB-fold refers to a fold-related function, which is able to adapt easily to binding a range of different biological molecules (Arcus 2002). The capacity of this structure to support many different sequences suggests evolutionary divergence of the OB-fold containing proteins having functional similarities. One of the most well-studied proteins with OB-fold are the single-stranded (ss) DNA-binding (Ssb) proteins.

Ssb proteins bind with ssDNA through their N-terminus OB-fold with high affinity in a sequence-independent manner (Lohman and Ferrari 1994). They have evolved to preserve ssDNA formed during DNA replication, recombination and repair. In addition to their role in DNA-binding, they also associate with a broad range of cellular genome maintenance proteins via the acidic C-terminal tail (Shereda et al. 2008). The positively charged N-terminus is separated from the negatively charged C-terminus by a 60 amino acid long region rich in proline and glycine residues (P/G-rich) (Lohman and Ferrari 1994).

Enhanced Ssb expression was observed upon DNA damage in *Escherichia coli* (Brandsma et al. 1983) as well as in *Bacillus subtilis* (Lindner et al. 2004). Even in the radiation-resistant bacterium, *Deinococcus radiodurans,* the 32 kDa Ssb was induced in response to ionizing

radiation and mitomycinC, both at transcriptional and protein level (Ujaoney et al. 2010), and was shown to play a major role in RecA-promoted DNA exchange reactions (Eggington et al. 2004). The bacterial *ssb* gene is found to be essential, and its deletion always results in lethality. Point mutations and temperature-sensitive mutations in *ssb* rendered *E. coli* cells extremely sensitive to UV, and they also exhibited decreased SOS induction and recombination (Carlini et al. 1998; Glassberg et al. 1979). Overexpression of Ssb, on the other hand, led to a decrease in the basal level of LexA and thereby an increase in the expression of *lexA*-controlled genes. Availability of excess Ssb increased the efficiency of excision repair, but decreased that of post-replication recombinational repair (Moreau 1987) and inhibited recombination of UV-irradiated bacteriophage DNA in *E. coli* (Moreau 1988).

The nitrogen-fixing cyanobacterium, *Anabaena* sp. strain PCC7120 (hereafter referred to as *Anabaena* 7120) has been shown to be resistant to high doses of γ-irradiation ($LD_{50} = 6$ kGy) (Singh et al. 2010), which is indicative of an efficient DNA repair system. Ssb protein, which forms the core of DNA repair system in all bacteria, is relatively unexplored in cyanobacteria. In the genome of *Anabaena* 7120 (http://genome.microbedb.jp/cyanobase), two ORFs have been annotated as *ssb*-like genes, namely *alr0088* and *alr7579*. However, the proteins encoded by these genes resemble C-terminal truncated Ssb proteins, possessing only the N-terminal OB-fold region which enabled them to bind ssDNA tested by in vitro biochemical assays, but in a manner different from other bacterial Ssbs (Kirti et al., unpublished results). Absence of the C-terminal acidic tail precludes interaction with other genome maintenance proteins and this raises a question as to whether the Alr0088 and Alr7579 proteins of *Anabaena* can function as true 'Ssb' proteins.

To answer this question, regulation of expression in vivo and the physiological role of Alr0088 and Alr7579 in response to DNA damage inducing stresses were analyzed in *Anabaena* 7120. Our data show that both the proteins are expressed in *Anabaena* under control conditions and differentially expressed under DNA damage inducing stress conditions. Overexpression of the two proteins individually in *Anabaena* 7120 has no significant effect on normal growth, but that of Alr0088 modulates tolerance to different DNA-damaging stresses.

Materials and methods

Organism and growth conditions

Escherichia coli cells were grown in Luria–Bertani (LB) medium in the presence of appropriate antibiotics at 37 °C with shaking at 150 rpm. The antibiotics used were 50 μg kanamycin mL^{-1} (Kan_{50}), or 100 μg carbenicillin mL^{-1}(Cb_{100}). Axenic cultures of *Anabaena* strains were grown in BG-11 liquid medium, pH 7.0 in the presence (BG-11, N^+) or absence (BG-11, N^-) of 17 mM $NaNO_3$, as a source of combined nitrogen (Castenholz 1988), under stationary conditions with continuous illumination (30 μE m^{-2} s^{-1}) at 27 °C ± 2 °C. Growth was assessed in terms of chlorophyll *a* (chl*a*) content of culture as described earlier (McKinney 1941). Cell survival was assessed by plating 100 μL of 10 μg chl*a* density mL^{-1} on to BG-11 (N^+/N^-) agar plates followed by incubation under illumination for 10 days. *Anabaena* strains harbouring the different plasmids [AnpAM: *Anabaena* harbouring pAM1956 (Rajaram and Apte 2010), An*alr0088*$^+$: *Anabaena* harbouring the plasmid pAM*alr0088* and An*alr7579*$^+$: *Anabaena* harbouring the plasmid pAM*alr7579*] were grown in the presence of 10 μg neomycin mL^{-1} (Nm_{10}) in BG-11 liquid media and 25 μg neomycin mL^{-1} (Nm_{25}) on BG-11 agar plates.

For all experiments, 3-day-old *Anabaena* cultures were concentrated to 10 μg chl*a* density mL^{-1} prior to subjecting them to stress. The different stresses applied were (i) mitomycinC stress with different concentrations of the mutagen (stock 1 mg mL^{-1} in water) for 30 min, (ii) UV stress by plating equal amount of culture on BG 11 (N^+/N^-) agar plates (Neo_{25} for *Anabaena* strains harbouring pAM1956-based plasmid), followed by exposure to UV-B (280 nm) (dose rate 5 J m^{-2} s^{-1}) for varying doses followed by incubation under continuous illumination and survival assessed after 10 days, and (iii) radiation stress of 6 kGy ^{60}Co γ-rays (dose rate 4.5 kGy h^{-1}). These were compared with unstressed cultures grown at 27 ± 2 °C. For experiment involving post-irradiation recovery (PIR) from irradiation stress, stressed and unstressed cultures were harvested, washed three times with fresh medium and inoculated in fresh growth medium at a chl*a* density of 1 μg mL^{-1} and incubated under usual growth conditions for 7 days.

Western blotting and immuno-detection

Ni–NTA affinity purified Alr0088 and Alr7579 proteins overexpressed in *E. coli* were used to raise the corresponding polyclonal antibodies (anti-Alr0088Ab and anti-Alr7579Ab) in rabbit. The two antibodies were very specific and showed no cross-reactivity, tested using pure proteins. Proteins from 3-day-old *Anabaena* 7120 cultures were extracted in Laemelli's buffer, electrophoretically separated on 14 % SDS–polyacrylamide gels followed by electroblotting on to nitrocellulose membrane. Alr0088 and Alr7579 proteins were immunodetected by using the corresponding antibody, followed by secondary anti-rabbit antibody tagged to alkaline phosphatase and colour development using NBT–BCIP.

Construction of *Anabaena* strains harbouring replicative plasmids

The ~0.34 kb (*alr0088*) and ~0.38 kb (*alr7579*) fragments (Genbank Accession nos. GU225949 and GU225950 for *alr0088* and *alr7579*, respectively) digested with *Nde*I-*Bam*HI were ligated individually to pFPN vector (Chaurasia et al. 2008) resulting in pFPN*alr0088*/pFPN*alr7579* plasmid constructs. The 0.59 and 0.62 kb *SalI–XmaI* fragments from these constructs were ligated to a stable shuttle vector pAM1956 (Yoon and Golden 1998), resulting in plasmid constructs designated as pAM*alr0088* and pAM*alr7579*. In these plasmid constructs, the individual genes were transcriptionally fused with downstream *gfp-mutII* gene, and co-transcribed from the light-inducible promoter P*psbA1*. These plasmids were introduced into *Anabaena* 7120 by conjugation and completely segregated *Anabaena* strains harbouring the plasmids were selected as described earlier (Raghavan et al. 2011).

Results

Effects of DNA-damaging agents on expression of Alr0088 and Alr7579 in *Anabaena*

The nitrogen-fixing cultures of *Anabaena* 7120 express both Alr0088 and Alr7579 under normal growth conditions (Fig. 1). Expression levels of Alr0088 and Alr7579 were measured using replicate gels. Levels of Alr0088 (panel I) were enhanced 1.4 ± 0.05-fold in response to mitomycinC (Fig. 1a) and UV-B stress (Fig. 1b), but was unchanged either immediately after exposure to 6 kGy of γ-irradiation (Fig. 1c) or during PIR (Fig. 1d). On the other hand, expression of Alr7579 (panel II) showed a dose-dependent effect in response to mitomycinC as well as UV-B stress, being enhanced 1.4 ± 0.05-fold upon exposure to 3 μg mitomycinC mL^{-1} for 30 min (Fig. 1c) or 1.2 ± 0.06-fold 3 kJ of UV-B (Fig. 1d). Though, only a 1.1 ± 0.03-fold increase in Alr7579 was observed immediately after irradiation (Fig. 1c), about twofold increase in level was observed during PIR (Fig. 1d). Expression of Alr0088 and Alr7579 under control growth conditions and their regulation in response to abiotic stresses under nitrogen-supplemented conditions were similar to that under nitrogen-fixing conditions (data not shown).

Overexpression of Alr0088 and Alr7579 proteins in *Anabaena* 7120

The *Anabaena* Alr0088 and Alr7579 proteins were individually overexpressed *in trans* from the light-inducible *psbA1* promoter using the plasmids pAM*alr0088* and pAM*alr7579* (Fig. 2a), respectively, in *Anabaena* 7120. Since, the *Anabaena* strains, An*alr0088*$^+$ and An*alr7579*$^+$ were grown continuously under constant illumination; the corresponding proteins along with GFP were constitutively expressed and the filaments exhibited green fluorescence (upon excitation with light of wavelength 470 nm), unlike wild type *Anabaena* 7120 which exhibited red fluorescence (data not shown). Monitoring of GFP expression was important to know the extent of segregation in these strains. Western blotting and immunodetection with the anti-Alr0088 and anti-Alr7579 antibodies confirmed the overexpression of the 13 kDa Alr0088 and 14 kDa Alr7579 in the *Anabaena* strains, An*alr0088*$^+$ and An*alr7579*$^+$, respectively (Fig. 2b). Overexpression of one protein did not affect the native levels of the other protein (data not shown).

Effect of overexpression of Alr0088 and Alr7579 on stress tolerance of *Anabaena*

MitomycinC stress

Anabaena cells were exposed to different concentrations of mitomycinC for a maximum of 30 min. In addition to the general effect of DNA adduct formation upon exposure to mitomycinC, *Anabaena* filaments also exhibited different degrees of fragmentation. Under normal growth conditions, the average filament size of 3-day-old cultures grown under stationary conditions was 108 ± 23 for both the *Anabaena* strains. The average filament length after exposure to 2 and 4 μg mL^{-1} of mitomycinC for 30 min was 16 ± 4 and 8 ± 3, respectively, for *Anabaena* 7120 as well as AnpAM, 28 ± 4 and 20 ± 3, respectively, for An*alr0088*$^+$, and 22 ± 3 and 14 ± 2, respectively, for An*alr7579*$^+$ irrespective of N-status, suggesting decreased fragmentation upon overexpression of the two proteins especially Alr0088. *Anabaena* cells under control conditions fragmented to 20–40 cell stage by mild sonication gave two- to threefold higher colony forming units (CFUs) than that shown for un-sonicated control cultures shown in Fig. 3 (data not shown). Under nitrogen-fixing conditions, the cell survival of AnpAM decreased to 69.08 and 61.17 %, respectively, upon exposure to 2 and 4 μg mL^{-1} of mitomycinC (Fig. 3a) and to about 65.15 and 49 %, respectively, under N-supplemented conditions (Fig. 3b). Under nitrogen-fixing conditions, the survival of An*alr7579*$^+$ and An*alr0088*$^+$, was comparable or marginally better than that of AnpAM cells (Fig. 3a). Under N-supplemented conditions, An*alr0088*$^+$ survival was superior at 4 μg mL^{-1} of mitomycinC compared to that of the other two strains (Fig. 3b). In spite of mitomycinC stressed AnpAM cells

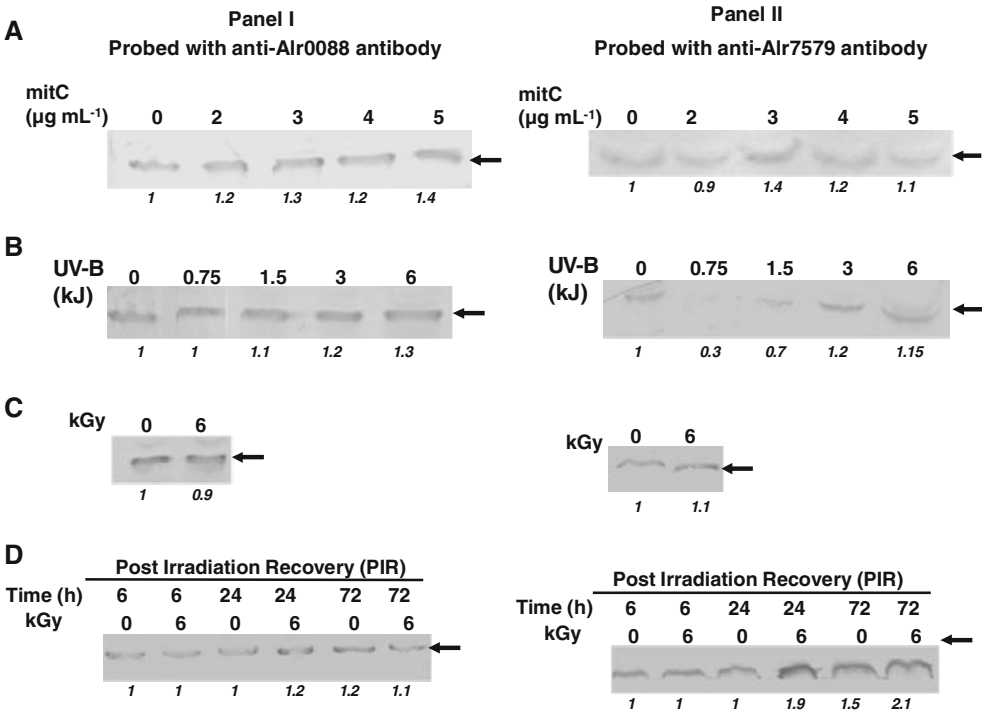

Fig. 1 Stress-induced change in expression level of Alr0088 (*panel I*) and Alr7579 (*panel II*) in *Anabaena* 7120. Three-day-old cultures of *Anabaena* 7120 were concentrated to 10 μg chl*a* density mL^{-1} and subjected to different stresses. Samples were drawn **a** after mitomycinC exposure (0–5 μg mL^{-1}) for 30 min, **b** UV-B exposure (0–3 kJ), **c** immediately (Imm) after 6 kGy of Co^{60} γ-irradiation, and **d** during post-irradiation recovery (PIR). Protein extracts (40 μg/lane) were electrophoretically separated by 14 % SDS-PAGE, electroblotted on to nitrocellulose membrane and probed with either anti-Alr0088 and anti-Alr7579 antibodies. The cross-reacting proteins Alr0088 (*panel I*) and Alr7579 (*panel II*) are indicated by *arrows*. The fold change in expression of Alr0088/Alr7579 levels with respect to that under control conditions (*1st lane*) was determined by densitometry and is shown below each gel

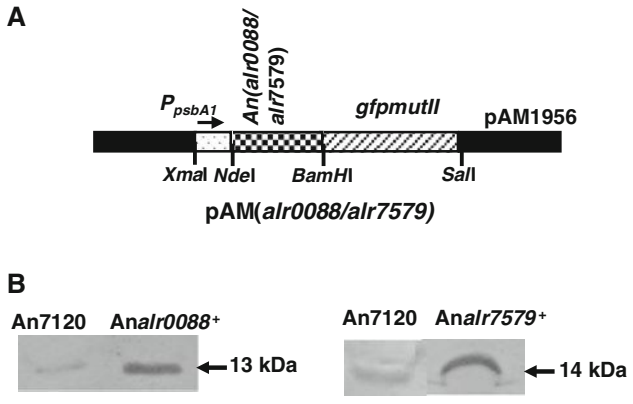

Fig. 2 **a** Schematic representation of the plasmid construct pAM*alr0088*/pAM*alr7579* used for overexpression of the corresponding proteins in *Anabaena* 7120. Restriction enzymes used are indicated. **b** Immunodetection of Alr0088 and Alr7579 in 3-day-old wild type *Anabaena* 7120, An*alr0088*$^+$, and An*alr7579*$^+$ cultures. Other details were as described in legend to Fig. 1

having shorter filaments and hence higher number of filaments per μg chl*a*, compared to An*alr0088*$^+$ and An*alr7579*$^+$ cells, the lower CFU indicated that in the absence of constitutive expression of either *alr0088* or *alr7579,* the cells more vulnerable to mitomycinC.

UV-B stress

Under nitrogen-fixing conditions, the survival of AnpAM and An*alr0088*$^+$ was comparable up to a dose of 1.5 kJ. Upon exposure to 3 kJ of UV-B, no colonies corresponding to AnpAM were detected, while An*alr0088*$^+$ and An*alr7579*$^+$ strains exhibited 4.8 % and 2.4 % survival, respectively (Fig. 3c). AnpAM cells were comparatively more susceptible to UV stress under N-supplemented conditions compared to nitrogen-fixing cultures (Fig. 3c, d). In contrast, the survival of An*alr0088*$^+$ and An*alr7579*$^+$ was better than AnpAM especially at 0.75 kJ of UV exposed (Fig. 3d).

γ-Irradiation stress

As compared to 50–55 % survival for AnpAM cells after exposure to 6 kGy of γ-irradiation irrespective of N-status (Fig. 4a), that for An*alr7579*$^+$ was marginally better (60 %) under both the conditions, in contrast, An*alr0088*$^+$ showed unusual radiosensitivity, with its survival declining to about 5 and 20 % under nitrogen-fixing and nitrogen-supplemented conditions, respectively (Fig. 4a). When allowed to recover after irradiation, both AnpAM and

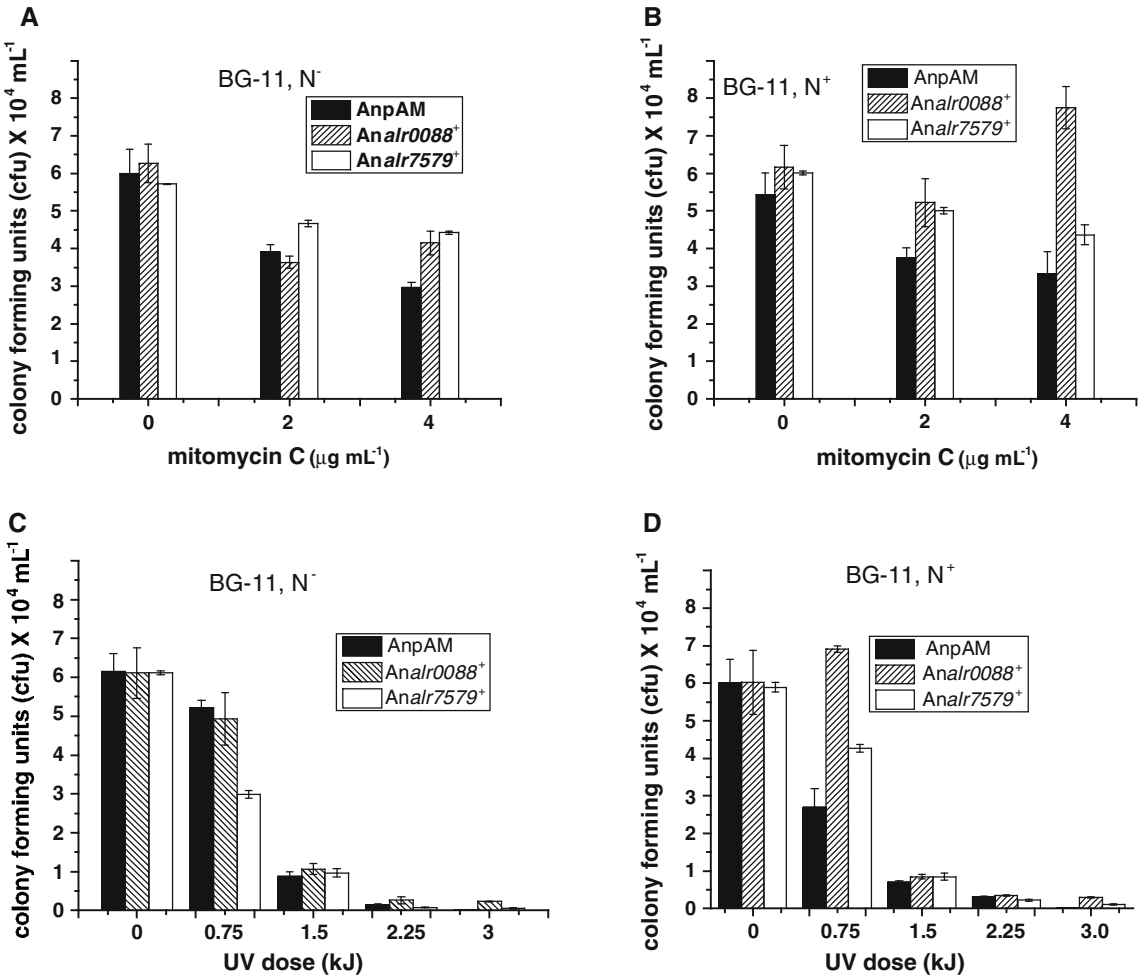

Fig. 3 Tolerance of *Anabaena* strains to stresses inducing DNA adduct formation. 3-day-old cultures of AnpAM, An*alr0088*$^{+}$, and An*alr7579*$^{+}$ grown in **a**, **c** nitrogen-fixing or **b**, **d** nitrogen-supplemented conditions were concentrated to 10 μg chl*a* density mL^{-1} (**a**, **b**). The concentrated cultures were subjected to different concentrations of mitomycinC (0–4 μg mL^{-1}) for 30 min. After the stress, cells were immediately plated on BG11 N^{-}/N^{+} agar plates supplemented with Neo$_{25}$. **c**, **d** Aliquots of 100 μl of concentrated cultures were spread on the corresponding **C** BG-11, N^{-} or **d** BG-11, N^{+} agar plates, followed by exposure to different doses of UV-B (0–3 kJ). The plates **a–d** were incubated under normal growth conditions and illumination. Colony forming units (CFUs) were counted at the end of 10 days

An*alr7579*$^{+}$ showed comparable recovery (Fig. 4b, c) after 7 days. While for An*alr0088*$^{+}$, recovery was only 35 % under nitrogen-fixing conditions (Fig. 4b) and 62 % under nitrogen-supplemented conditions (Fig. 4c).

Discussion

Prokaryotic Ssb proteins are generally 17–18 kDa in size comprising of over 160 amino acids having three characteristic regions of N-terminal OB-fold, P/G-rich region, and a C-terminal acidic tail. The smallest reported bacterial Ssb proteins (TmaSsb and TneSsb) (Olszewski et al. 2010) comprising of about 140 amino acids has all the three regions, though shorter in length. On the other hand, the annotated Ssb proteins (Alr0088 and Alr7579) of *Anabaena* 7120 are much smaller, having only a little over 100 amino acids and possess only the N-terminal OB-fold, rendering them as the smallest known Ssb-like proteins reported till date. Only PriB proteins, structurally similar to Ssb and speculated to have originated by gene duplication (Ponomarev et al. 2003), has only the N-terminal OB-fold similar to Alr0088 and Alr7579. The presence of OB-fold, which signifies the ability to bind ss-DNA, is not unique to Ssb-like proteins. OB-fold is found in RecG, Termination factor Rho, few ribosomal proteins and t-RNA synthetases and some bacterial toxins (Theobald et al. 2003). Thus, the presence of OB-fold is not indicative of a Ssb, but of the capability of proteins to bind ss-DNA. Indeed our recent studies based on inherent aromatic acid fluorescence quenching experiments have shown that Alr0088 binds to small oligo nucleotides of

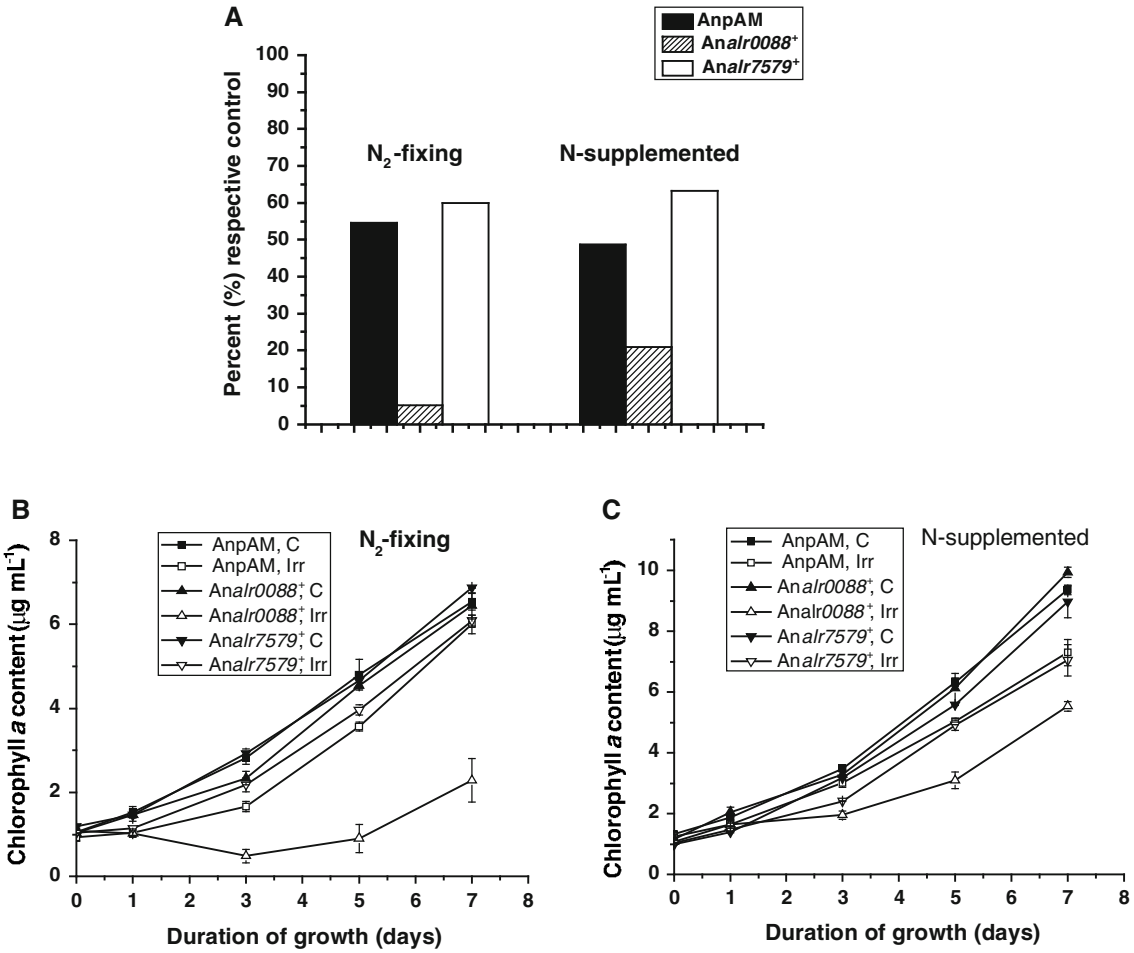

Fig. 4 Tolerance of *Anabaena* strains to stresses inducing ss and ds DNA breaks. Three-day-old cultures of AnpAM, An*alr0088*$^{+}$, and An*alr7579*$^{+}$ grown under **a**, **b** nitrogen-fixing or **a**, **c** nitrogen-supplemented conditions were concentrated to 10 μg chl*a* density mL^{-1} and exposed to 6 kGy of ^{60}Co γ-irradiation. **a** Survival was measured in terms of colony forming units immediately after irradiation and compared with the respective un-irradiated (sham) control. **b**, **c** For recovery, the stressed and control cultures were washed, inoculated in fresh BG-11, N^{-}/N^{+}, $Neo_{12.5}$ liquid media at 1 μg chl*a* density mL^{-1} and allowed to grow under normal growth conditions for 7 days. Growth during post-irradiation recovery was measured in terms of chlorophyll *a* content over a 7-day period

50–60 bases, while Alr7579 binds to large ssDNA (Kirti et al., unpublished results).

In *E. coli*, a twofold increase in Ssb level was seen after exposure to very high doses of mitomycinC (Moreau 1987). Such increase was slow compared to RecA and did not result in significant increase in its levels (Perrino et al. 1987). In the radioresistant bacterium, *D. radiodurans*, Ssb levels were enhanced 2.6-fold in response to mitomycinC stress and fivefold during PIR (Ujaoney et al. 2010), but exhibited no change in response to UV and desiccation stresses. In *Anabaena* 7120, expression of both the annotated Ssb-like proteins, was modulated in response to the DNA damage inducing stresses tested, however, the fold increase was not very significant.

Exposure to both mitomycinC and UV-radiation are known to evoke SOS-response in *E. coli* and other bacteria which is regulated through the LexA transcriptional repressor (Janion 2008). However, of the two genes, *alr0088* and *alr7579*, a LexA-binding box is found upstream of only *alr0088* (Mazon et al. 2004). The difference in the promoter/regulatory regions of *alr0088* and *alr7579* could be the reason for their differential expression in response to SOS-inducing stresses (Fig. 1a, b). Of the two genes, expression of '*alr7579*' seems to be regulated more significantly in response to DNA-damaging stresses compared to '*alr0088*' (Fig. 1). This, however, had no bearing on the stress tolerance achieved upon constitutive overexpression of the two proteins individually in *Anabaena*. In fact, An*alr0088*$^{+}$ overexpressing Alr0088 protein exhibited different degree of tolerance to various stresses compared to wild type *Anabaena* 7120 or AnpAM cells, while overexpression of Alr7579 in An*alr7579*$^{+}$ did not have a very significant bearing on stress tolerance of *Anabaena*.

An*alr0088*$^+$ cells exhibited higher tolerance to both mitomycinC and UV-B stress compared to vector control, AnpAM as well as Alr7579 overexpressing An*alr7579*$^+$ cells (Fig. 3). The best protection observed was under N-supplemented conditions, with survival up by 50 % when exposed to either 4 μg mitomycinC mL^{-1} for 30 min (Fig. 3b) or 0.75 kJ of UV-B (Fig. 3d) upon overexpression of Alr0088 compared to empty vector control, AnpAM. When exposed to mitomycinC, the An*alr0088*$^+$ cells showed least fragmentation of filaments compared to other two strains. Since chlorophyll *a* content of the different cultures plated before or after mitomycinC stress remains unchanged, the number of filaments would determine the CFUs and by this logic, *Anabaena* 7120 and An*alr7579*$^+$ exposed to mitomycinC stress should exhibit higher CFUs. However, the reverse was observed indicating that in spite of having lower number of filaments per μl of culture plated, the viability of An*alr0088*$^+$ filaments were higher, resulting in higher CFUs. Studies in Archae bacteria revealed that protection against DNA adduct formation could be achieved by Rpa C (Ssb) even in the absence of the C-terminal tail (Skowyra and Macneil 2012), and this along with the ability of Alr0088, but not Alr7579, to bind small stretches of ssDNA (Kirti et al., unpublished results) protects them from nucleolytic digestion and thereby allows better protection upon overexpression of the protein in *Anabaena.*

On the other hand, γ-irradiation which causes DNA damage in the form of single- and double-stranded breaks (Henner et al. 1983), binding of Alr0088 to the short ssDNA stretches would protect them from nucleolytic digestion, but due to the absence of C-terminal tail, they would not be able to recruit DNA repair proteins and proceed towards repair pathway. As shown recently in Archae bacteria, in the absence of C-terminal, RpaC does not offer protection against single- and double-stranded breaks formed upon exposure to Phleomycin C (Skowyra and Macneil 2012). Thus, in An*alr0088*$^+$ cells, free ssDNA may not be available to participate in repair process thereby increasing the radiosensitivity of these cells. Alr7579, on the other hand, does not bind short stretches of ssDNA (Kirti et al., unpublished results) and thus may not be interfering with the normal DNA repair process in *Anabaena*, thereby exhibiting tolerance comparable to wild type cells. The differential effect towards tolerance to DNA damage inducing stresses observed upon individual overexpression of the two Ssb proteins, suggest that the two *ssb*-like genes are not redundant. The inability to generate viable knock out strains of either *alr0088* or *alr7579* in *Anabaena* (Kirti et al., unpublished results) suggested that the genes were essential.

Of the two Ssb-like proteins of *Anabaena*, neither may function as a full-fledged Ssb due to the truncation of C-terminal tail. The Alr0088 may function as a single-stranded DNA-binding protein by protecting small stretches of ssDNA, generated during the course of DNA damage or repair without taking part in the subsequent DNA repair process per se. The Alr7579 protein, on the other hand, may function as part of a large DNA-binding complex and thus its individual overexpression may not significantly contribute to tolerance of *Anabaena* to DNA-damaging stresses.

References

Arcus V (2002) OB-fold domains: a snapshot of the evolution of sequence, structure and function. Curr Opin Struct Biol 12:794–801

Brandsma JA, Bosch D, Backendorf C, Van de putte P (1983) A common regulatory region shared by divergently transcribed genes of the *Escherichia coli* SOS system. Nature 305:243–245

Carlini L, Curth U, Kindler B, Urbanke C, Porter RD (1998) Identification of amino acids stabilizing the tetramerization of the single-stranded DNA-binding protein from *Escherichia coli*. FEBS Lett 430:197–200

Castenholz RW (1988) Culturing of cyanobacteria. Methods Enzymol 167:68–93

Chaurasia AK, Parasnis A, Apte SK (2008) An integrative expression vector for strain improvement and environmental application of the nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC7120. J Microbial Methods 73:133–141

Eggington JM, Haruta N, Wood EA, Cox MM (2004) The single-stranded DNA binding protein of *Deinococcus radiodurans*. BMC Microbiol 4:2. doi:10.1186/1471-2180-4-2

Glassberg J, Meyer RR, Kornberg A (1979) Mutant single-strand binding protein of *Escherichia coli*: genetic and physiological characterization. J Bacteriol 140:14–19

Henner WD, Rodriquez LO, Hecht SM, Haseltine WA (1983) Gamma ray induced deoxyribonucleic acid strand breaks 3′ glycolate termini. J Biol Chem 258:711–713

Janion C (2008) Inducible SOS response system of DNA repair and mutagenesis in *Escherichia coli*. Int J Biol Sci 4:338–344

Lindner C, Nijland R, Van Hartskamp M, Bron S, Hamoen LW, Kuipers OP (2004) Differential expression of two paralogous genes of *Bacillus subtilis* encoding single-stranded DNA binding protein. J Bacteriol 186:1097–1105

Lohman TM, Ferrari ME (1994) *Escherichia coli* single-stranded DNA binding protein: multiple DNA-binding modes and cooperativities. Annu Rev Biochem 63:527–570

Mazon G, Lucena JM, Campoy S, Fernandez de Henestrosa, Candau P, Barbe J (2004) LexA binding sequences in Gram-positive and cyanobacteria are closely related. Mol Gen Genomics 271:40–49

McKinney G (1941) Absorption of light by chlorophyll solutions. J Biol Chem 140:315–322

Moreau PL (1987) Effects of overproduction of single-stranded DNA-binding protein on RecA protein-dependent processes in *Escherichia coli*. J Mol Biol 194:621–634

Moreau PL (1988) Overproduction of single-stranded DNA binding protein specifically inhibits recombination of UV-irradiated bacteriophage DNA in *Escherichia coli*. J Bacteriol 170: 2493–2500

Olszewski M, Grot A, Wojciechowski M, Novak M, Mickiewicz M, Kur J (2010) Charcterization of exceptionally thermostable single-stranded DNA binding proteins from *Thermotoga maritima* and *Theromotoga neapolitana*. BMC Microbiol 10:260–270

Perrino FW, Rein DC, Bobst AM, Meyer RR (1987) The relative rate of synthesis and levels of single-stranded DNA binding protein during induction of SOS repair in *Escherichia coli*. Mol Gen Genet 209:612–614

Ponomarev VA, Makarova KS, Aravind L, Koonin EV (2003) Gene duplication with displacement and rearrangement: origin of the bacterial replication protein PriB from the single-stranded DNA-binding protein, Ssb. J Mol Microbiol Biotechnol 5:225–229

Raghavan PS, Rajaram H, Apte SK (2011) Nitrogen status dependent oxidative stress tolerance conferred by overexpression of MnSOD and FeSOD proteins in *Anabaena* sp. strain PCC7120. Plant Mol Biol 77:407–417

Rajaram H, Apte SK (2010) Differential regulation of *groESL* operon expression in response to heat and light in *Anabaena*. Arch Microbiol 192:729–738

Shereda RD, Kozlov AG, Lohman TM, Cox MM, Keck JL (2008) SSB as an organizer/mobilizer of genome maintenance complexes. Crit Rev Biochem Mol Biol 43:289–318

Singh H, Fernandes T, Apte SK (2010) Unusual radioresistance of nitrogen-fixing cultures of *Anabaena* strains. J Biosci 35: 427–434

Skowyra A, Macneil SA (2012) Identification of essential and non-essential single-stranded DNA binding proteins in a model archeal organism. Nucleic Acids Res 40:1077–1090

Theobald DL, Mitton-Fry RM, Wuttke Carlini DS (2003) Nucleic acid recognition by OB-fold proteins. Annu Rev Biophys Biomol Struct 32:115–133

Ujaoney AK, Potnis AA, Kane P, Mukhopadhyaya R, Apte SK (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 192:5637–5644

Yoon HS, Golden JW (1998) Heterocyst pattern formation controlled by a diffusible peptide. Science 282:935–938

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The Hypothetical Protein 'All4779', and Not the Annotated 'Alr0088' and 'Alr7579' Proteins, Is the Major Typical Single-Stranded DNA Binding Protein of the Cyanobacterium, *Anabaena* sp. PCC7120

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Abstract

Single-stranded DNA binding (SSB) proteins are essential for all DNA-dependent cellular processes. Typical SSB proteins have an N-terminal Oligonucleotide-Binding (OB) fold, a Proline/Glycine rich region, followed by a C-terminal acidic tail. In the genome of the heterocystous nitrogen-fixing cyanobacterium, *Anabaena* sp. strain PCC7120, *alr0088* and *alr7579* are annotated as coding for SSB, but are truncated and have only the OB-fold. *In silico* analysis of whole genome of *Anabaena* sp. strain PCC7120 revealed the presence of another ORF '*all4779*', annotated as a hypothetical protein, but having an N-terminal OB-fold, a P/G-rich region and a C-terminal acidic tail. Biochemical characterisation of all three purified recombinant proteins revealed that they exist either as monomer or dimer and bind ssDNA, but differently. The All4779 bound ssDNA in two binding modes i.e. $(All4779)_{35}$ and $(All4779)_{66}$ depending on salt concentration and with a binding affinity similar to that of *Escherichia coli* SSB. On the other hand, Alr0088 bound in a single binding mode of 50-mer and Alr7579 only to large stretches of ssDNA, suggesting that All4779, in all likelihood, is the major typical bacterial SSB in *Anabaena*. Overexpression of All4779 in *Anabaena* sp. strain PCC7120 led to enhancement of tolerance to DNA-damaging stresses, such as γ-rays, UV-irradiation, desiccation and mitomycinC exposure. The tolerance appears to be a consequence of reduced DNA damage or efficient DNA repair due to increased availability of All4779. The ORF *all4779* is proposed to be re-annotated as *Anabaena ssb* gene.

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Introduction

Single-stranded DNA-binding proteins (SSB) are ubiquitous proteins found in all bacteria. The SSB proteins are characterised by their non-specific binding to single-stranded DNA (ssDNA) and active participation in the maintenance of genome integrity (DNA repair) as well as genetic information transfer (replication and transcription) [1]. A typical SSB monomer consists of (a) an N-terminal region having several conserved residues responsible for binding to ssDNA, tetramerisation and stabilization of monomer fold [2], and (b) a C-terminal region which displays a low sequence conservation except for the last few amino acids (known as acidic tail), and is responsible for protein-protein interactions and recruitment of DNA interactive proteins [3]. The highly conserved OB-fold has been extensively described for *Escherichia coli* SSB protein [1]. Deletion of C-terminus diminishes recruitment of other DNA interacting proteins [3], but enhances the affinity of N-terminus to ssDNA [4]. The spacer region between the N-terminal OB-fold and C-terminal acidic tail is rich in proline/glycine (P/G) residues and is thought to modulate the strength of DNA binding, possibly by distancing the highly negatively charged C-terminal end from the positively charged DNA binding N-terminal domain [5].

Nitrogen-fixing cyanobacteria, such as strains of *Anabaena* and *Nostoc* exhibit tolerance to a variety of abiotic stresses such as salinity, desiccation, heat and radiation [6,7], strongly indicative of a robust mechanism of DNA repair in these microbes [8]. Unfortunately, genes/proteins involved in DNA metabolism of cyanobacteria have not received adequate attention. The SSB protein is a key protein involved in all DNA related cellular activities. In the genomic database of *Anabaena* sp. strain PCC7120 (hereafter referred to as *Anabaena* 7120), two ORFs '*alr0088*' and '*alr7579*' are annotated as coding for SSB-like proteins (http://genome.microbedb.jp/cyanobase/Anabaena) and exhibit 28–30% homology at amino acid level with EcoSSB, and about 42% homologous to each other. However, BLAST search [9] of the amino acid sequence of these two proteins show that the protein sequence is terminated immediately after the N-terminal OB-fold region and have no region corresponding to either the P/G spacer or the C-terminal acidic tail. Since, C-terminal acidic tail is essential for interaction with other DNA replication/repair/recombination proteins [3], it seems unlikely that the proteins encoded by these two ORFs can perform all the functions of SSB proteins. However, this does not rule out that they are genuine SSBs, since the second SSB (SsbB) of naturally transformable bacteria, such as *Bacillus subtilis*, lacks the C-terminal acidic tail,

Table 1. Primers used and PCR amplicons generated in this study.

Primers	Nucleotide Sequence*	R.E.	Amplicon#
*alr0088*Fwd	5′ GGCCATATGAGCATTAACATTGTC 3′	*Nde*I	*alr0088* ORF (0.35 kb)
alr0088 Rev	5′ GGCGGATCCTTAAAAATTTTCTGGTGC 3′	*Bam*HI	
*alr7579*Fwd	5′ GGCCATATGAACTATATCAACAAA 3′	*Nde*I	*alr7579* ORF (0.38 kb)
alr7579 Rev	5′ GGCGGATCCCTAGAAATTTGCGTTAGC 3′	*Bam*HI	
*all4779*Fwd	5′ GGCCATATGAACAGCTGTGTTTTA 3′	*Nde*I	*all4779* ORF (0.55 kb)
all4779 Rev	5′ GGCGGATCCTAAAATGGAATATCGTC 3′	*Bam*HI	

*The restriction endonuclease (R.E.) site included in each primer is underlined and the corresponding R.E. indicated in the adjacent column.
#The amplicons generated with a given set of PCR primers are specified.
doi:10.1371/journal.pone.0093592.t001

but functions as a SSB and is involved in genetic recombination [10,11]. Alr0088 and Alr7579 exhibit about 36–38% overall homology with BsSsbB. An *in silico* search for a SSB-like protein with the C-terminal region (i.e. the spacer region and acidic tail) in the genome of *Anabaena* 7120, revealed the ORF '*all4779*'. The ORF encodes a 182 amino acid long protein with an N-terminal OB-fold, a P/G rich spacer region and a C-terminal acidic tail but has been annotated as a hypothetical protein possibly due to its lower homology (15–18%) with other bacterial SSBs. The homologs of the two truncated SSB-like proteins as well as hypothetical SSB-like protein of *Anabaena* 7120 are found across all cyanobacterial genomes (http://genome.microbedb.jp/cyanobase).

In the present work, we cloned, overexpressed, purified and biochemically characterised Alr0088, Alr7579 and All4779 proteins. All three proteins existed in monomeric and dimeric forms and showed differential binding to ssDNA. All4779 protein showed typical structural features, oligomerisation, ssDNA binding modes compared to *E. coli* SSB and conferred tolerance to DNA damage, upon overexpression in *Anabaena*, that identifies it as the major SSB of *Anabaena*.

Materials and Methods

Organism and Growth Conditions

E. coli cells were grown in Luria–Bertani (LB) medium at 37°C with shaking (150 rpm). When required antibiotics [34 μg chloramphenicol mL^{-1}(Cm_{34}), 50 μg kanamycin mL^{-1} (Kan_{50}), or 100 μg carbenicillin mL^{-1} (Cb_{100})] were used in culture media. Axenic cultures of *Anabaena* 7120 were grown in BG-11 liquid medium, pH 7.0 [12] in the absence of combined nitrogen (BG-11, N^-) under stationary conditions with continuous illumination (30 μE m^{-2} s^{-1}) at 27°C±2°C. Recombinant *Anabaena* strains were grown in the presence of 10 μg neomycin mL^{-1} (Nm_{10}) in BG-11 liquid media or with 25 μg neomycin mL^{-1} (Nm_{25}) on BG-11 agar plates. Growth was assessed in terms of chlorophyll *a* content per ml of culture as described earlier [13]. Cell survival was assessed in terms of colony forming units (cfu) by plating 100 μl of the culture on to BG-11, N^- agar plates followed by incubation under illumination for 10 days as described earlier [14].

Three-day-old nitrogen-fixing *Anabaena* cultures were concentrated to a chlorophyll *a* density of 10 μg mL^{-1}, prior to subjecting them to one of the following stresses: (i) 6 kGy of ^{60}Co γ-rays at a dose rate of 4.5 kGy h^{-1}, or (ii) 6 days of desiccation or in humid chamber (control), or (iii) 0–4 μg mitomycinC (mitC) mL^{-1} for 30 min, or (iv) exposure to 0–1.5 kJ UV-B (280 nm) (dose rate 5 J m^{-2} sec^{-1}) for different duration. Survival in response to stress, and post-stress recovery were compared with unstressed/control cultures grown under illumination at 27°C±2°C as described earlier [14].

Generation of Plasmid Constructs for Overexpression of Proteins in *E. coli*

Different amplicons (*alr0088*, *alr7579*, *all4779*) were generated by PCR amplification of *Anabaena* 7120 genomic DNA (100 ng) using gene specific primers (as indicated in Table 1), 1 μM dNTPs and 1U Taq DNA polymerase in Taq buffer (Bangalore Genei, India). These amplicons were individually digested with *Nde*I and *Bam*HI restriction endonucleases and ligated to the expression

Table 2. Plasmids used in this study.

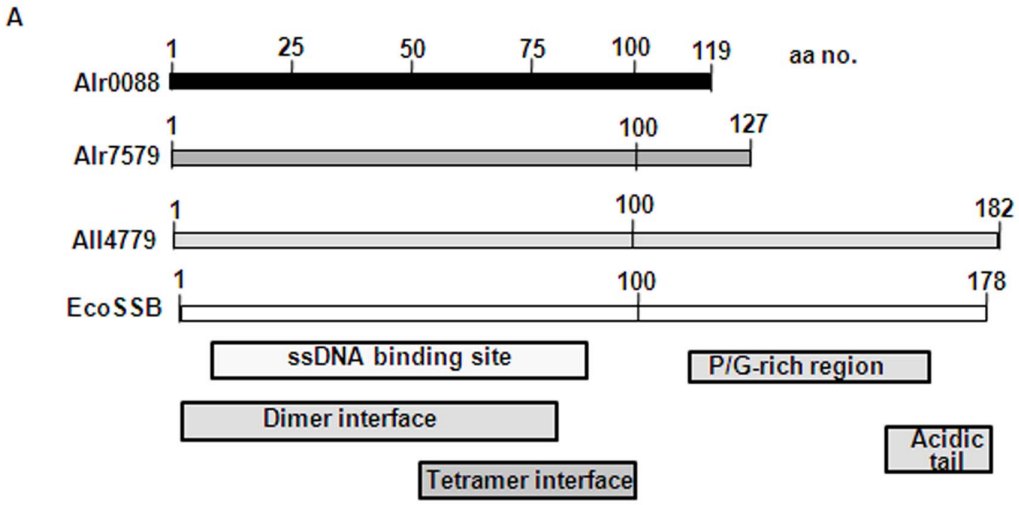
Plasmids	Description	Source/Reference
pET16b	Cb^r, expression vector	Novagen
pAM1956	Kan^r, promoterless vector with *gfpmut2* reporter gene	[19]
pBluescript (pBS)	Cb^r, cloning vector	Lab Collection
pFPN	Cb^r, Kan^r, integrative expression vector	[18]
pET*alr0088*	Cb^r, 0.35 kb *alr0088* gene cloned in pET16b at *Nde*I/*Bam*HI restriction sites	This study
pET*alr7579*	Cb^r, 0.38 kb *alr7579* gene cloned in pET16b at *Nde*I/*Bam*HI restriction sites	This study
pET*all4779*	Cb^r, 0.55 kb *all4779* gene cloned in pET16b at *Nde*I/*Bam*HI restriction sites	This study
pFPN*all4779*	Cb^r, Kan^r, 0.55 kb *all4779* gene cloned in pFPN at *Nde*I/*Bam*HI restriction sites	This study
pAM*all4779*	Kan^r, 0.81 kb *Xma*I-*Sal*I fragment from pFPN*all4779* gene cloned in pAM1956	This study

doi:10.1371/journal.pone.0093592.t002

Table 3. Bacterial strains used in this study.

Bacterial Strains	Description	Source/ Reference
E. coli strains		
DH5α	F⁻*recA*41 *endA*1 *gyrA*96 *thi*-1*hsd*R17 (rk^-mk^-) *supE*44 *relA* λ Δ*lacU*169	Lab Collection
HB101	F⁻mc^r Bm^r rhsdS20($r_B^-m_B^-$) *recA*13 *leuB*6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rps (Sm^R) *gln*V44 λ⁻	Lab Collection
BL21(pLysS)	Cm^r F⁻*ompT* hSdSB ($r_B^-m_B^-$) *gal dcm* pLysS (pLysS) (DE3)	Novagen
BL21(pLysS)(pET*alr0088*)	Cm^r, Cb^r, *E. coli* BL-21 cells harbouring the plasmid, pET*alr0088*	This study
BL21(pLysS)(pET*alr7579*)	Cm^r, Cb^r, *E. coli* BL-21 cells harbouring the plasmid, pET*alr7579*	This study
BL21(pLysS)(pET*all4779*)	Cm^r, Cb^r, *E. coli* BL-21 cells harbouring the plasmid, pET*all4779*	This study
Ec(pAM*all4779*)	Kan^r, HB101 harbouring pAM*all4779* plasmid	This study
HB101 (pRL623+ pRL443)	Donor strain carrying pRL623 (encoding methylase) and conjugal plasmid pRL443	(Wolk, C.P.)
Anabaena strains		
Anabaena 7120	Wild type strain	Lab Collection
AnpAM	Nm^r, *Anabaena* 7120 harbouring the plasmid, pAM1956	[26]
An*all4779*$^+$	Nm^r, *Anabaena* 7120 harbouring the plasmid, pAM*all4779*	This study

doi:10.1371/journal.pone.0093592.t003



B

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All4779   1 M-----NSCVLLAEIIQEPQLRYTSDNLAVTEMLVQFPNSQKPDDPPATLK-------VVGWGNL  53
            M     N  +L+  + Q+P++RY  +  AV  + +    S + D     +K       VV +G L
EcoSSB    6 MASRGVNKVILVGNLGQDPEVRYMPNGGAVANITLATSESWR-DKATGEMKEQTEWHRVVLFGKL  64

All4779  54 ATEIQQNYHQGDRVIIAGRLGMNTIDRPEGFKEKRAELTVQQIQSISGSGNFTSSPAVSRTPAVTE  119
            A    +   +G +V I G+L      R     +  +           + G  T   +++R
EcoSSB   65 AEVASEYLRKGSQVYIEGQL------RTRKWTDQSGQDRYTTEVVVNVGG--TMQMLGGR--QGGG  120

All4779 120 TPSRPPSPAVTTQHDLPSYESPRPAPTPAPSKVSVPPQVNNYEP-APQTTYSPVEVP-DP-DDIPF 182
             P                                                    E P D  DDIPF
EcoSSB  121 APAGGNIGGGQPQGGWGQPQQPQGGNQFSGGAQSR-PQQSA--PAAPSN-----EPPMDFDDDIPF 178
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Figure 1. Bioinformatic analysis of Alr0088, Alr7579 and All4779 proteins of *Anabaena* 7120. (A) Conserved Domain Database (CDD) analysis of *Anabaena* Alr0088, Alr7579 and All4779 proteins and *E. coli* SSB (EcoSSB) protein. The OB-fold corresponding ssDNA binding region, dimer and tetramer interfaces for all the proteins are indicated. (B) Comparison of homology between predicted amino acid sequence of All4779 and EcoSSB. The identical amino acids are indicated by letters and similar amino acids with a '+' sign. The proline (P) and glycine (G) residues beyond the OB fold are shown in larger font, while the acidic residues at the C-terminal end are in bold and underlined. The numbers on the left and right hand side correspond to amino acid residues.
doi:10.1371/journal.pone.0093592.g001

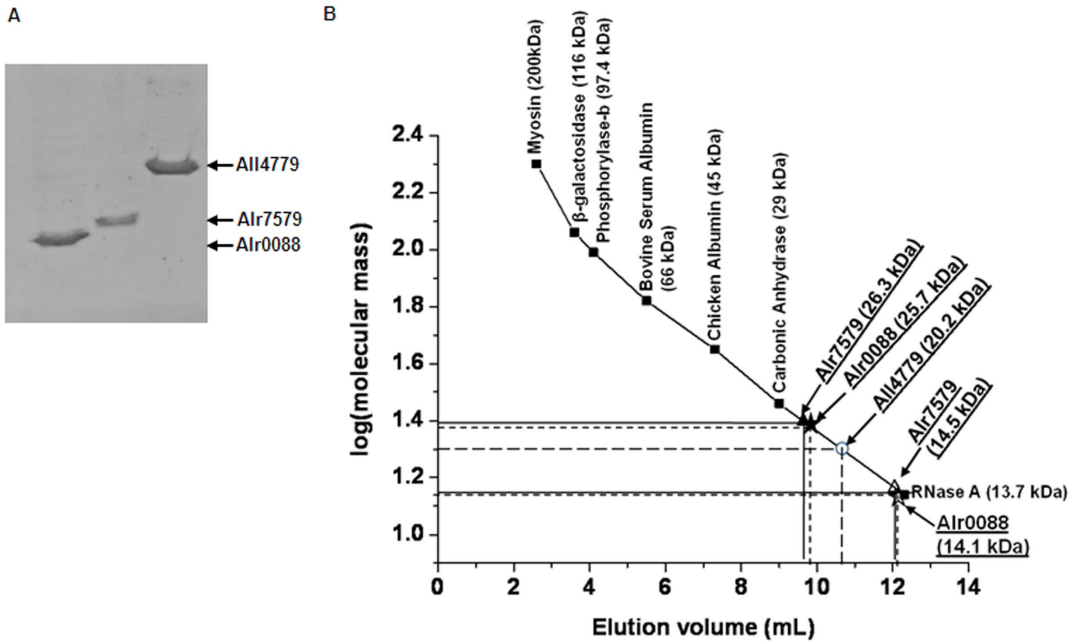

Figure 2. Molecular mass determination of purified native *Anabaena* proteins. (A) Ni-NTA affinity chromatography purified Alr0088, Alr7579 and All4779 proteins separated on 12% SDS-polyacrylamide gel followed by staining with Coomassie Brilliant Blue (CBB) G-250. The purified proteins are indicated by arrows. (B) Elution profile of purified native Alr0088, Alr7579 and All4779 proteins in gel filtration chromatography using Superdex HR200 matrix. A standard graph using the following standard proteins: [Myosin (200 kDa), β-galactosidase (116 kDa), Phosphorylase-b (97.4 kDa), Bovine Serum Albumin (66 kDa), Chicken Albumin (45 kDa), Carbonic Anhydrase (29 kDa) and RNaseA (13.7 kDa)] was drawn to calculate the molecular mass of the eluted native *Anabaena* proteins depending on their elution volume. The position of the eluted proteins has been depicted by 'star' and 'triangle' symbols. The vertical and horizontal lines from the two symbols indicate the elution volume and the corresponding log of molecular mass.
doi:10.1371/journal.pone.0093592.g002

vector pET16b (Table 2), having His_{10}-tag at the 5′ end, at identical restriction sites. The resulting plasmid constructs were designated as pET*alr0088*, pET*alr7579* and pET*all4779* respectively (Table 2). DNA insert of all three plasmids were sequenced on both strands using Sanger's dideoxy method and were found to be completely identical to the corresponding nucleotide sequences available in the genomic database. The nucleotide sequences corresponding to *alr0088*, *alr7579* and *all4779* respectively were submitted to GenBank (GenBank Accession Nos. GU225949, GU225950, GU225951).

Overexpression and Purification of His-tagged Proteins

The plasmids pET*alr0088*, pET*alr7579* and pET*all4779* (Table 2) were transformed into *E. coli* BL21(pLysS) and transformants selected on $LBCm_{34}Cb_{100}$ plates (Table 3). Proteins were overexpressed from the respective logarithmic phase cultures of *E. coli* upon addition of 1 mM IPTG for 3 h at 37°C. The recombinant His-tagged proteins (Alr0088, Alr7579 and All4779) were extracted in lysis buffer (20 mM Tris-HCl, pH 8, 0.5 M NaCl, 5 mM imidazole and 0.1% TritonX-100) by sonication and purified by Ni-NTA affinity chromatography (Qiagen, Germany) using different concentrations of imidazole ranging from 10–1000 mM as described earlier. The proteins were eluted individually in 1 M imidazole fraction and visualised by electrophoretic separation on 14% SDS-polyacrylamide gel followed by staining with Coomassie Brilliant Blue (CBB) G-250. The proteins were quantified spectrophotometrically by measuring absorbance at 280 nm using 19480 M^{-1} cm^{-1}, 20970 M^{-1} cm^{-1} and 12950 M^{-1} cm^{-1} as the extinction coefficients, estimated using Expasy software (*web.expasy.org/protparam*) for Alr0088, Alr7579 and All4779 proteins respectively.

The purified recombinant native proteins were individually cross-linked using glutaraldehyde, as described earlier [15], followed by precipitation of protein with cold acetone. The pellet was air dried, solubilised in 1X Laemmli's buffer by heating at 80°C for 10 min, separated by SDS-PAGE and visualized by staining with CBB G-250.

Molecular mass determination of native purified proteins was carried out by gel filtration chromatography using Superdex HR200 column. The coumn was equilibrated with Tris-NaCl buffer and standard graph obtained using the following proteins: Myosin (200 kDa), β-galactosidase (116 kDa), Phosphorylase-b (97.4 kDa), Bovine Serum Albumin (66 kDa), Chicken Albumin (45 kDa), Carbonic Anhydrase (29 kDa) and RNase A (13.7 kDa). Molecular mass of the three *Anabaena* proteins was calculated from the standard graph on the basis of the elution volume. Presence of protein in different eluates/fractions was detected by measuring absorbance at 280 nm.

Electrophoretic Mobility Shift Assay (EMSA)

A 75-mer oligonucleotide (10 ng) was end-labelled with γ-^{32}P-ATP using Polynucleotide Kinase. The labelled oligo was incubated in the presence of specified concentrations of the Alr0088, Alr7579 and All4779 proteins in binding buffer [20 mM Tris-HCl, pH 8, 1 mM $MgCl_2$, 100 mM KCl, 8 mM Dithiothreitol (DTT), 4% sucrose, 80 μg mL^{-1} Bovine Serum Albumin (BSA)] for 30 min at room temperature and electrophoretically separated subsequently on 6% non-denaturing polyacrylamide gel at 150 V for 2 h in 1X Tris-borate EDTA (TBE) buffer. Imaging of the radioactive gel was carried out using Phosphorimager Typhoon Trio Variable mode imager (Wipro-GE-HealthCare, USA).

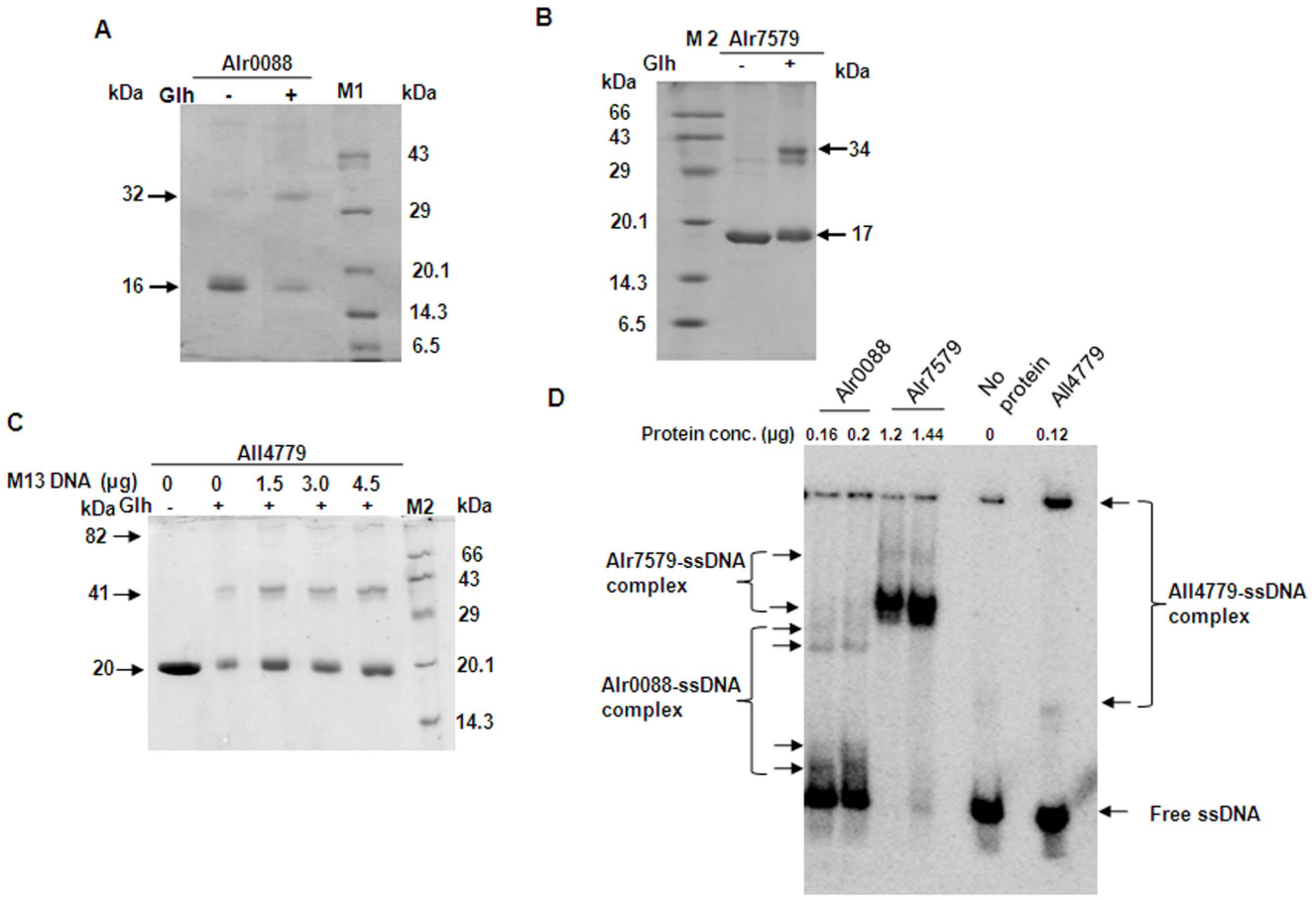

Figure 3. Glutarldehyde (Glh)-aided crosslinking of native purified *Anabaena* SSB-like proteins and their binding to ssDNA. The purified native *Anabaena* proteins (A) Alr0088, (B) Alr7579 and (C) All4779 were cross-linked with Glh in the presence or absence of M13 ssDNA as indicated. The proteins were electrophoretically separated on 12% SDS-polyacrylamide gel followed by staining with Coomassie Brilliant Blue (CBB) G-250. The molecular mass of the protein markers used (M1 and M2) are written to the immediate (right/left) of the marker lane. Different molecular forms of the native *Anabaena* proteins are indicated by the arrows. (D) Electrophoretic Mobility Shift Assay (EMSA) of a γ-^{32}P-ATP labeled 75-mer oligonucleotide in the presence of different concentrations of Alr0088, Alr7579 and All4779 proteins. Following *in solution* interaction, the assay mix was separated by 6% non-denaturing PAGE in 1X TBE and radioactive gel imaged using a phosphorimager. The free ssDNA substrate and the different ssDNA-protein complexes formed are indicated.
doi:10.1371/journal.pone.0093592.g003

Fluorescence Measurements

All three proteins showed maximum excitation at 282 nm. The emission maxima were found to be 310, 340 and 335 nm respectively for Alr0088, Alr7579 and All4779 proteins in 20 mM Tris-HCl, pH 8, 1 mM EDTA buffer. The change in the intensity of the emitted fluorescence was measured in the presence of increasing concentration of ssDNA [poly(dT) or M13 ssDNA]. The graph of relative fluorescence (%) as a function of poly(dT) concentration was used to determine the binding constant for the individual proteins to ssDNA as described earlier [16]. The binding constants were calculated as the reciprocal of the concentration of poly(dT) at which 50% fluorescence compared to the initial 100% was detected. The graph depicting quenching expressed as the ratio of difference in fluorescence to initial fluorescence ($\Delta F/F_i$) as a function of the ratio of concentrations of poly(dT) and protein was used to determine the binding modes or occlusion site of the proteins as described earlier [17]. It corresponded to the $[nt]_{poly(dT)}/[Protein]$ value at the point of saturation of quenching of fluorescence. During titration, solutions were added from concentrated samples and correction for dilution was made as required. All fluorescence measurements were performed with Jasco spectrofluorimeter FP6500 (Japan) using a quartz cuvette of 1 cm path length at room temperature.

Western Blotting and Immuno-detection

The purified All4779 protein was used to raise specific polyclonal antibody (anti-All4779 antibody) in rabbit. Proteins were extracted from three-day-old wild type and recombinant *Anabaena* cultures in Laemmli's buffer, separated by 14% SDS-PAGE followed by electroblotting on to nitrocellulose membrane. Immunodetection was carried out using the 1:5000 dilution of anti-All4779 antibody, followed by secondary anti-rabbit IgG antibody, coupled to alkaline phosphatase and colour development using NBT-BCIP.

Generation of Recombinant *Anabaena* Strains

The 0.55 kb *Nde*I-*Bam*HI fragment from pET*all4779* was ligated to pFPN vector (Table 2) [18] at the same restriction sites, resulting in the plasmid construct, pFPN*all4779*. The 0.81 kb *Sal*I–*Xma*I fragment from pFPN*all4779* was ligated to pAM1956 vector (Table 2) [19] digested with the identical restriction enzymes. The

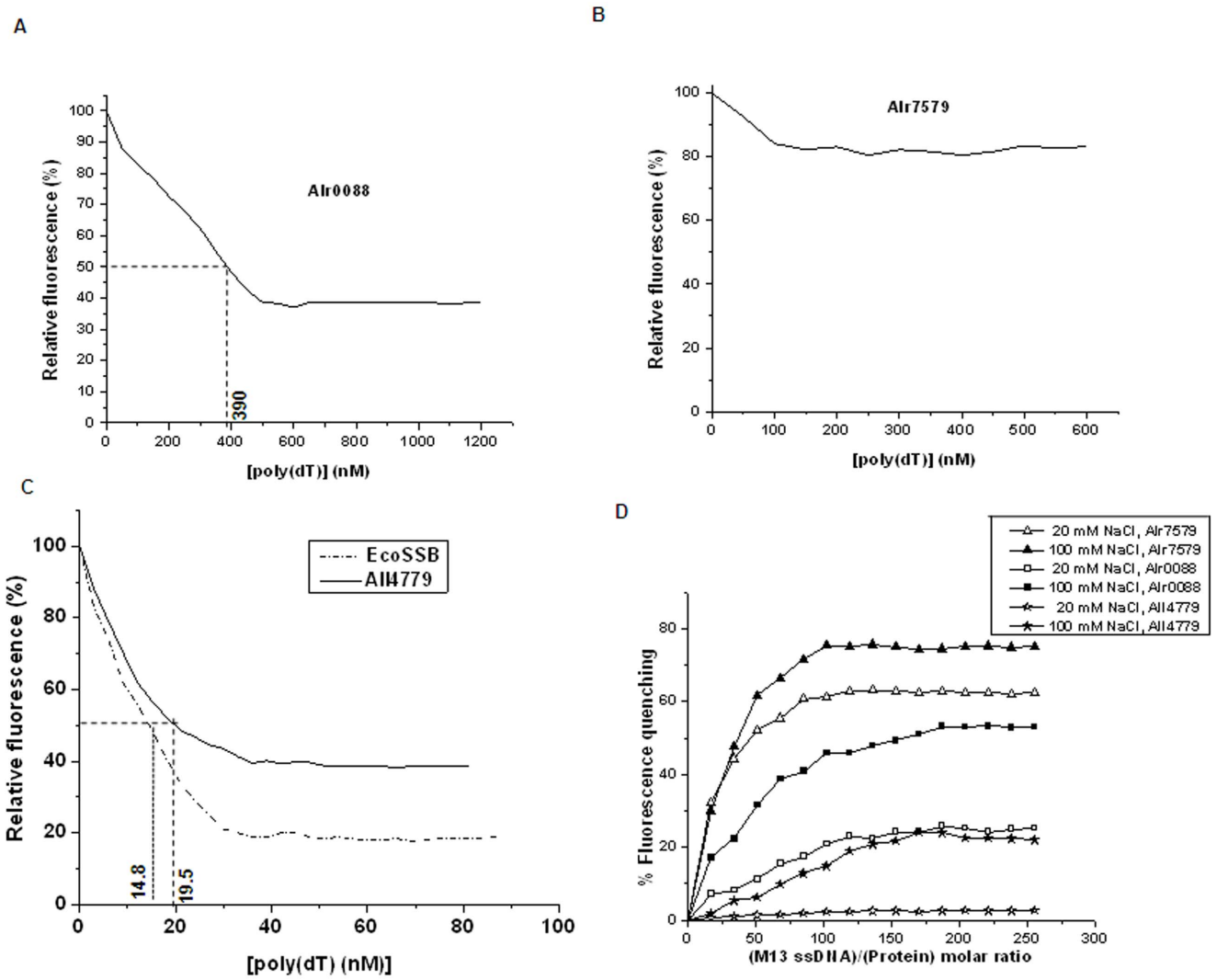

Figure 4. Relative quenching of fluorescence of native purified *Anabaena* SSB-like proteins and EcoSSB as a function of ssDNA concentration. (A–C) Quenching of fluorescence in 20 mM NaCl as a function of poly(dT) concentration of (A) Alr0088, (B) Alr7579 and (C) All4779 and purified EcoSSB (commercially available, Sigma) proteins represented as relative fluorescence, considering the observed fluorescence in the absence of any poly(dT) as 100%. The horizontal line designates the point on the graph wherein relative fluorescence is 50% and the corresponding vertical line indicates the concentration of poly(dT) at which it is achieved. Reciprocal of this concentration corresponds to the binding constant of the protein for poly(dT). (D) Percent fluorescence quenching of Alr0088, Alr7579 and All4779 proteins as a function of molar ratio of M13ssDNA and protein in the presence of 20 mM or 100 mM NaCl. The fluorescence quenching in the absence of M13ssDNA is considered as 0%.
doi:10.1371/journal.pone.0093592.g004

resulting construct was designated as pAM*all4779* (Table 2). In this construct, the *gfpmut2* gene (coding for Green Fluorescent Protein, GFP) is co-transcribed with the upstream *all4779* gene from the P_{psbA1} promoter, but the two transcripts are translated independently as described earlier [20]. Recombinant *Anabaena* strain overexpressing All4779 protein (An*all4779*$^{+}$) (Table 3) was generated by introducing the plasmid pAM*all4779* into *Anabaena* by conjugation as described earlier [20], and repeated selection on BG-11 agar plates containing 17 mM $NaNO_3$ (BG-11, N^{+}) and Neo_{25}, till completely segregated cells, uniformly expressing GFP, were obtained.

Results and Discussion

Bio-informatic Analysis of Alr0088, Alr7579 and All4779 Proteins

The *alr0088*, *alr7579* and *all4779* genes respectively encode 119, 127 and 182 amino acid long polypeptides (Figure 1A) with an estimated molecular mass of 13, 14 and 20 kDa. The prokaryotic SSBs are generally about 160–180 amino acids long, having a molecular mass of 17–18 kDa, except for SsbB of naturally transformable bacteria, which in case of BsSsbB is 113 amino acids long [11]. The SsbA protein of *B. subtilis* is 172 amino acids long, similar in size to EcoSSB [10]. Among the naturally non-transformable bacteria, the smallest known bacterial SSB are from the thermophilic bacteria, *Thermotoga maritima* (TmaSSB) and *T. neapolitana* (TneSSB) consisting of 141 and 142 amino acids respectively, having a single OB-fold domain and a C-terminal domain with the conserved DEPPF terminal amino acids [21].

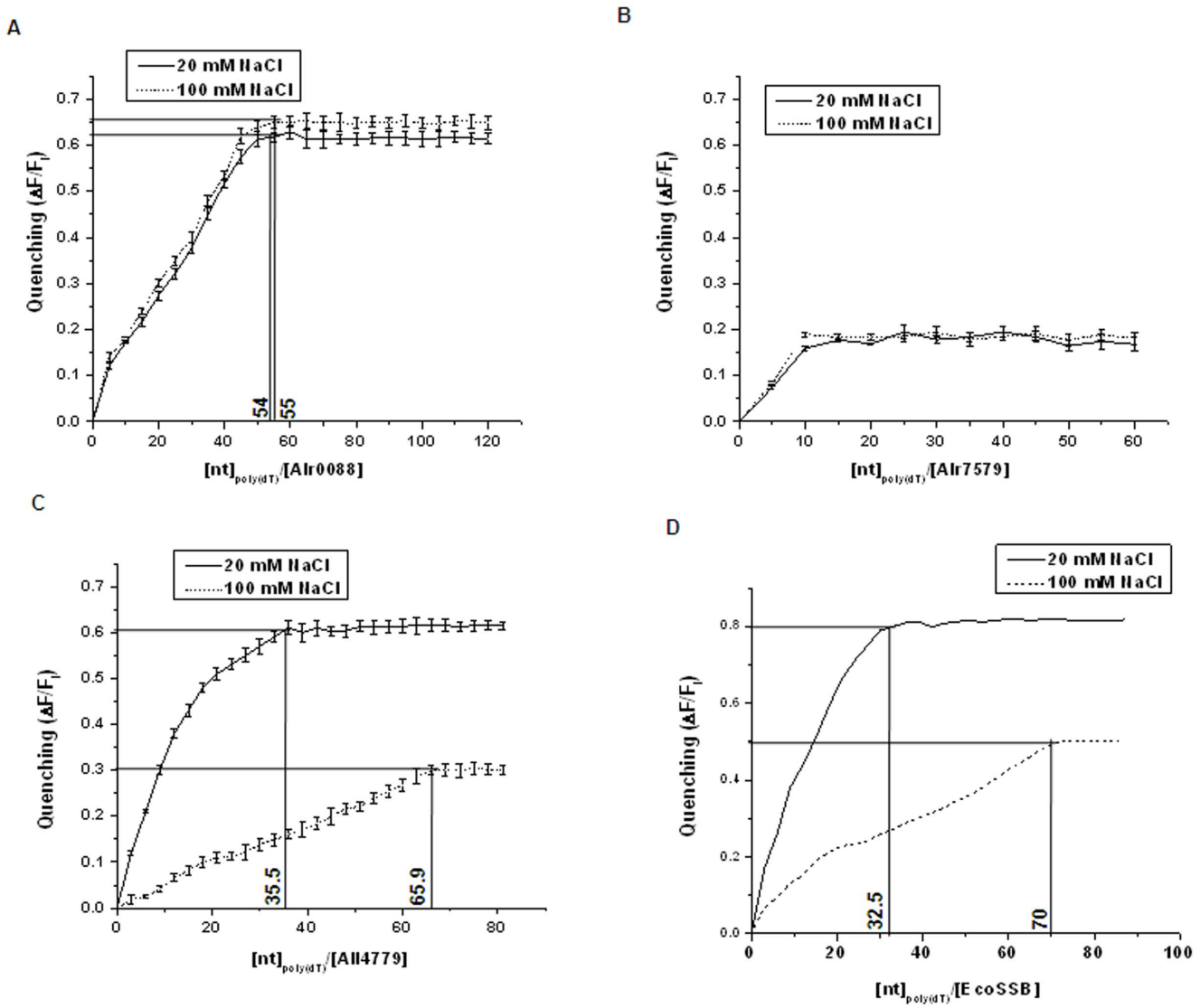

Figure 5. Quenching of fluorescence of native purified *Anabaena* SSB-like proteins compared with that of EcoSSB. The quenching of fluorescence of (A) Alr0088, (B) Alr7579, (C) All4779 and (D) EcoSSB in the presence of 20 mM or 100 mM NaCl was expressed as a ratio of change in fluorescence (ΔF) and initial fluorescence (F_i). The ($\Delta F/F_i$) was expressed as a function of ratio of concentrations of poly(dT) and protein. The horizontal lines indicate the point of saturation and the vertical lines drawn from the point of saturation indicate the probable length of ssDNA bound by one molecular unit of the protein.
doi:10.1371/journal.pone.0093592.g005

Among eukaryotes, the *Hs*mtSSB is 133 amino acids long and does not have the region corresponding to 1/3rd of the C-terminal region of EcoSSB [22]. Amino acid sequence analysis using Conserved Domain Database (CDD) [9] revealed the presence of a putative ssDNA-binding OB-fold domain and dimer/tetramer interface within N-terminal half in all three proteins similar to that in *E. coli* SSB (Figure 1A). All4779 additionally had a long C-terminal region similar to that observed for EcoSSB which comprised of a proline-rich region (19 residues) with two glycine residues, as compared to EcoSSB which is glycine rich (21 residues) and has 8 prolines in the corresponding region (Figure 1B). While multiple glycine residues allow flexibility in structure, multiple proline residues provide rigidity and kinks in the structure and thus no ordered structure results in gly-rich or pro-rich regions [23]. The proline-rich region of All4779 would also separate the positively charged N-terminal and the negatively charged C-terminal regions, similar to that in EcoSSB [5]. The N-terminal region of All4779 exhibited 26% identical and 48% similar amino acid residues and a nearly identical acidic tail compared to EcoSSB (Figure 1B). In spite of having an N-terminal OB-fold, P/G rich region and a C-terminal acidic tail, the low homology of All4779 to other known bacterial SSB proteins may possibly account for it not being annotated earlier as SSB-like protein in the genome database of *Anabaena* 7120, unlike Alr0088 and Alr7579 which show a greater homology than All4779 in the OB-fold region.

Biochemical Characterisation of *Anabaena* Alr0088, Alr7579 and All4779 Proteins

The *Anabaena* Alr0088, Alr7579 and All4779 proteins overexpressed in *E. coli* BL21(pLysS) cells were purified to near homogeneity using Ni-NTA affinity chromatography (Figure 2A).

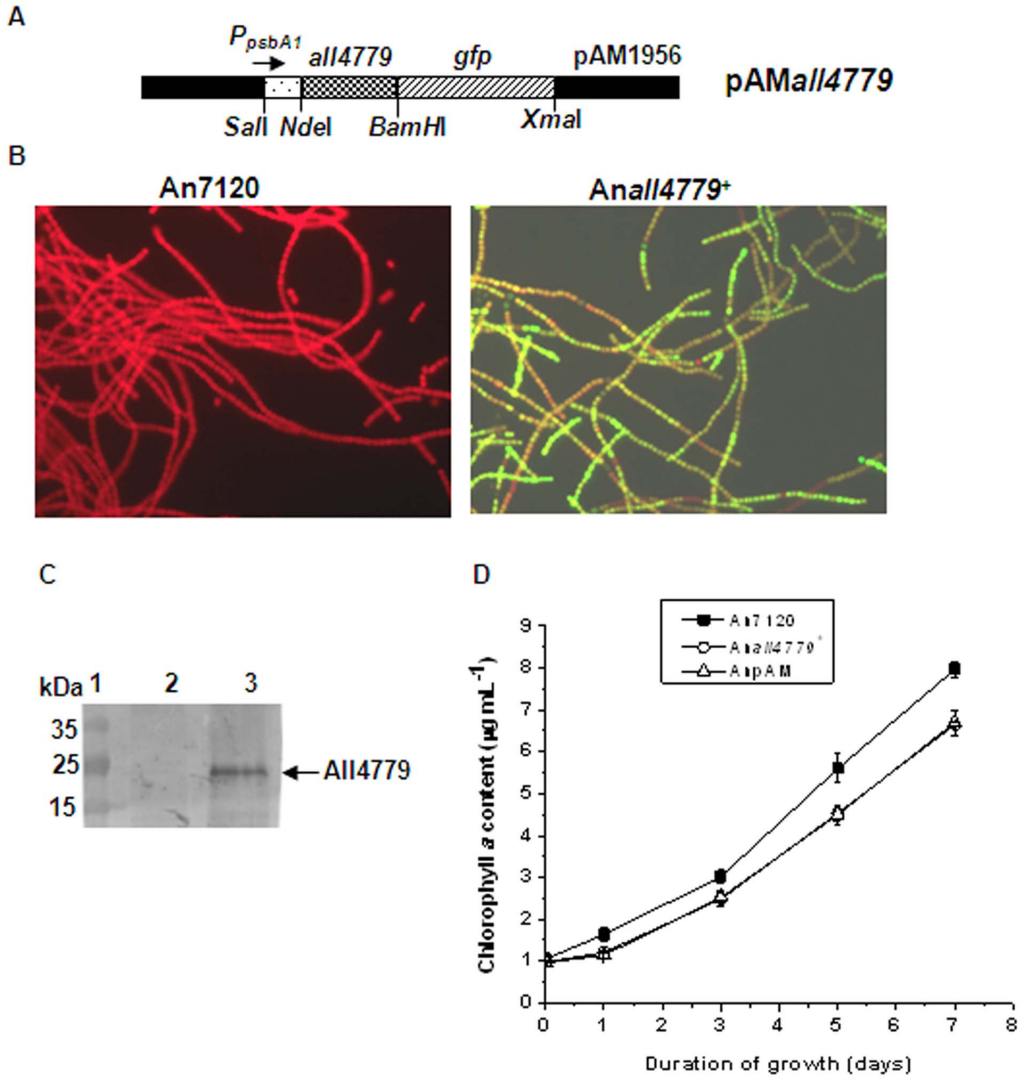

Figure 6. Construction of recombinant *Anabaena* strain overexpressing All4779 protein. (A) Schematic diagram of the plasmid construct, pAM*all4779* used for overexpression of All4779 protein in *Anabaena*. The different restriction enzymes used for cloning are indicated. (B) Fluorescence microphotograph (600X magnification) [using Hg-Arc lamp (excitation 470 nm, emission 508 nm)] of *Anabaena* 7120 [An7120] and recombinant strain, An*all4779*$^+$, grown for 3 days in BG-11, N^- media. (C) Protein extracts from *Anabaena* 7120 (lane 1) and An*all4779*$^+$ (lane 2) were separated by 12% SDS-PAGE, followed by blotting on to nitrocellulose membrane and immunodetection of All4779 protein using anti-All4779 antibody. The cross-reacting All4779 protein is indicated by an arrow. Equal loading controls are shown below the blot. Other details were as described in legend to Figure 2. (D) Growth profile of wild type *Anabaena* 7120 and recombinant *Anabaena* strain, An*all4779*$^+$ and AnpAM under nitrogen-fixing conditions over a period of 7 days. Growth was measured in terms of increase in chlorophyll *a* content. Recombinant strains were grown in presence of neomycin while wild type was grown without antibiotic.
doi:10.1371/journal.pone.0093592.g006

Presence of dimer and tetramer interfaces in the amino acid sequence (Figure 1A) suggested possibility of formation of multimers by the protein. Alr0088 and Alr7579 were eluted in two distinct fractions and All4779 in a single fraction (Figure 2B) upon separation by gel filtration chromatography using Superdex HR200. On the basis of elution profile of standard proteins on the same matrix, the molecular mass of the different fractions was predicted as 14.1 kDa and 25.7 kDa for Alr0088, 14.5 kDa and 26.3 kDa for Alr7579 and 20.2 kDa for All4779 (Figure 2B). This indicated dimerisation of Alr0088 and Alr7579 proteins as against only the monomeric form detected for All4779 protein. Higher molecular forms of these proteins were not detected even at higher protein concentrations (data not shown). This did not conform to the bioinformatic prediction for the three proteins which indicate the presence of dimeric and tetrameric interfaces (Figure 1A). Further probing of multimeric status was carried out by cross-linking the native proteins with glutaraldehyde followed by separation by SDS-PAGE. Upon cross-linking, the dimeric forms corresponding to 32 kDa for Alr0088 (Figure 3A), 34 kDa for Alr7579 (Figure 3B) and 41 kDa for All4779 (Figure 3C) were detected, with the levels of the dimeric form being lowest for All4779. This could be the reason for the inability to detect a higher molecular weight peak during gel-filtration chromatography for All4779 (Figure 2B). In the presence of M13 ssDNA the levels of the dimeric 41 kDa form as well as a probable tetrameric form of ~82 kDa increased (Figure 3C). This suggested that All4779 attains the native multimeric conformation preferably in the presence of ssDNA. In case of Alr0088 and Alr7579, no effect

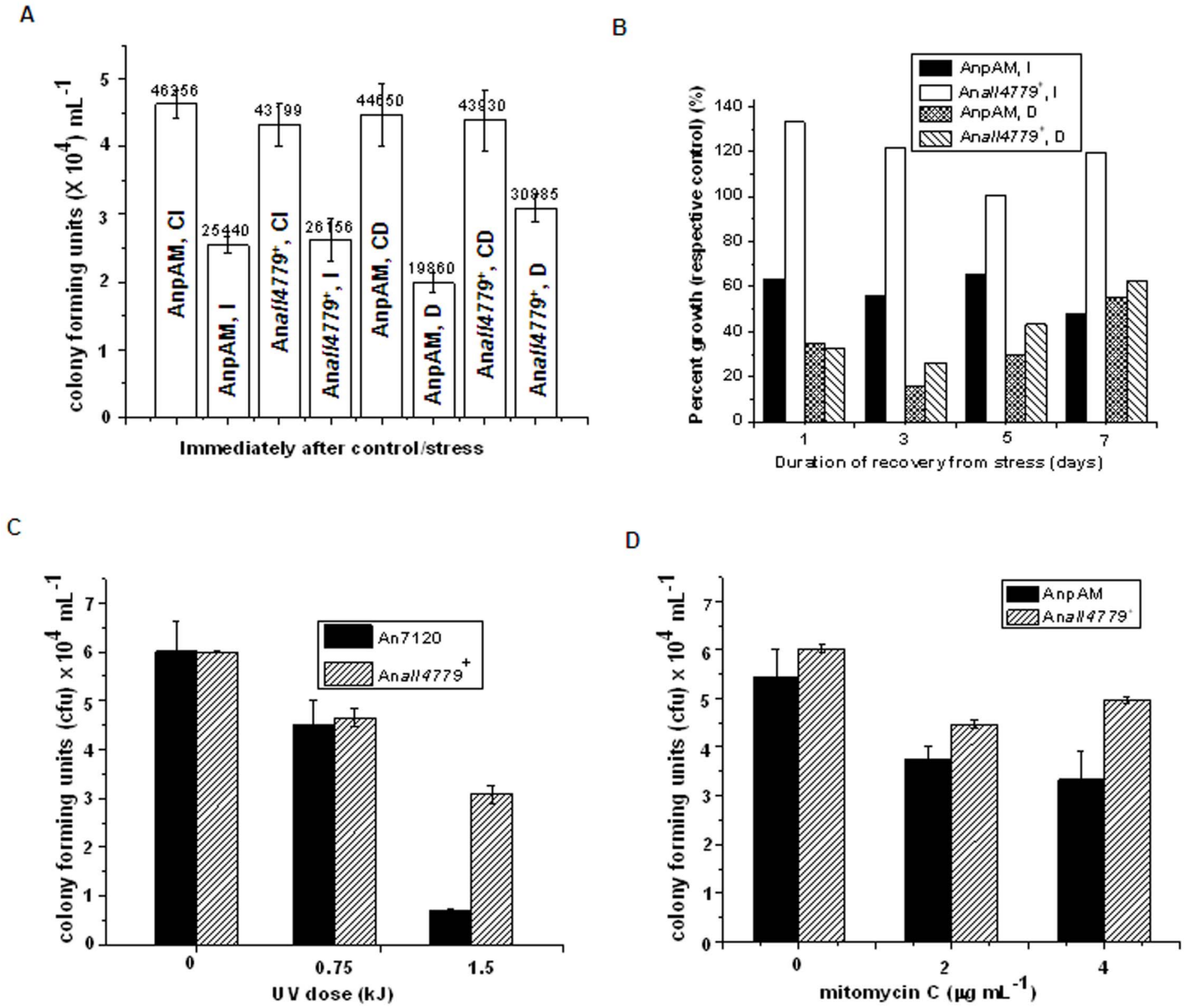

Figure 7. Effect of All4779 overexpression on the survival and tolerance of *Anabaena* to DNA-damage inducing stresses. (A and B) Three day-old cultures were concentrated to 10 µg chl*a* density mL^{-1} and exposed to 6 kGy of $^{6\circ}$Co γ-irradiation or to 6 days of desiccation. (A) Survival was measured in terms of colony forming units immediately after irradiation (I) or desiccation (D) and compared with the respective unirradiated control (CI) or undesiccated control (CD). (B) The stressed and control cultures were washed, inoculated in fresh BG-11, N^-, $Neo_{12.5}$ and allowed to recover under normal growth conditions for 7 days. Growth during post-irradiation/desiccation recovery was measured in terms of chlorophyll *a* content and expressed as percent of respective unirradiated/undesiccated controls. (C and D) Three-day-old cultures of recombinant strains AnpAM and An*all4779*$^+$ were concentrated to 10 µg chl*a* mL^{-1} density. (C) An 100 µl aliquot was spread on the corresponding BG-11, N^-, Neo_{25} agar plates and exposed to UV-B (0–1.5 kJ) (D) Culture aliquots were exposed to mitomycinC (0–4 µg ml^{-1}) for 30 min in liquid media followed by plating 100 µl on BG-11, N^- Neo_{25} agar plate. Colonies were counted after 10 days of incubation at 27°±2°C with constant illumination.
doi:10.1371/journal.pone.0093592.g007

on the levels of the dimeric form or generation of tetrameric form was observed even with ssDNA (data not shown). In general, bacterial SSB proteins function as tetramers [1], with the exception of thermophilic group of organisms (Thermus spp.) and the radioresistant microbe *Deinococcus radiodurans* [24] which function as dimer. However, the protomers of SSB of these organisms are twice the size of *E. coli* SSB and contain two OB-folds per monomer [24].

The DNA binding ability of the SSB-like proteins was assessed by Elctrophoretic Mobility Shift Assay (EMSA) and fluorescence quenching techniques. Multiple shifts in the mobility of the 75-mer ss oligonucleotide was observed in the presence of Alr0088 (Fig. 3D). Increase in concentration beyond 0.2 µg Alr0088 did not result in any further shifts in mobility (data not shown). Alr7579 decreased the mobility of the 75-mer oligo only when used at very high concentrations of 1.2–1.4 µg (Fig. 3D). Presence of Alr7579 also resulted in detection of multiple bands differing in their mobility, but majority of the complex formed, even when low concentrations of 0.12 µg of All4779 was used, was detected near the well (Fig. 3D). Based on this, the binding efficiency for ssDNA seems to be maximum for All4779, followed by Alr0088 and the least for Alr7579. The binding affinity for each of these proteins for ssDNA was calculated by fluorescence quenching technique using poly(dT) as the ssDNA substrate.

The relative fluorescence of native *Anabaena* proteins Alr0088, Alr7579 and All4779, was measured as a function of increasing

concentration of poly(dT) at 20 mM NaCl. The relative fluorescence of (i) Alr0088 decreased to a maximum of 40% with ~450 nM poly(dT) (Figure 4A), (ii) Alr7579 showed less than 20% decrease (Figure 4B), and (iii) All4779 up to 40% of the initial fluorescence, but at much lower concentrations (~35 nM) of poly(dT) (Figure 4C). Based on this, the binding constant, as an average of three independent experiments, was calculated as $2.56\pm0.4\times10^6$ M^{-1} for Alr0088, $5.13\pm0.71\times10^7$ M^{-1} for All4779 and 6.76×10^7 M^{-1} for EcoSSB (Figure 4C), which was comparable to that reported for EcoSSB ($5.5\pm1.5\times10^7$ M^{-1}) [4]. In the absence of C-terminal acidic tail, the binding affinity for ssDNA has been shown to increase 10-fold in case of EcoSSB [4], as well as for HsmtSSB, which lacks the C-terminal tail, calculated as 4×10^8 M^{-1} [25]. However, the reverse was found to be true in case of *Anabaena* 7120, with Alr0088 which lacks the acidic tail, having 10-fold lower binding affinity than All4779. This could be due to the additional absence of the P/G-rich region as well in Alr0088.

The inability of Alr7579 to bind poly(dT) raised questions on whether the OB-fold, responsible for binding ssDNA [1] is active in Alr7579. To test this, a larger ssDNA, such as M13 ssDNA was used as a substrate. A 60–70% quenching of the fluorescence of Alr7579 was observed with the 7 kb M13 ssDNA, the efficiency being higher at high NaCl concentration (Figure 4D), which allows formation of a more compact structure of ssDNA. The quenching of fluorescence of Alr7579 was not observed with thermally denatured M13 ssDNA (data not shown). M13 ssDNA is known to form secondary structures [26], which are disrupted at higher temperature. This suggested that Alr7579 may be recognising secondary structures formed with long ssDNA, rather than short stretches of linear ssDNA. Both Alr0088 and All4779 also bound M13 ssDNA at high salt concentration, but with lower efficiency, the quenching of fluorescence being 40% and 18% respectively (Figure 4D). While low quenching of fluorescence of Alr0088 by M13 ssDNA was observed at low NaCl (Figure 4D), indicating low level interactions, no such interaction was observed for All4779 (Figure 4D).

In general, SSB proteins interact with ssDNA in multiple binding modes, differing in the number of OB-folds which interact with the ssDNA. In the $(SSB)_{35}$ mode, approximately 35 nucleotides of ssDNA interact with two subunits of the Ssb tetramer, while in $(SSB)_{65}$ mode, ~65 nucleotides of ssDNA wrap around all four subunits, which is more favoured at higher salt concentrations [25]. Based on the quenching of fluorescence ($\Delta F/F_i$) of the three *Anabaena* proteins with poly(dT) at low (20 mM) NaCl and high (100 mM) NaCl concentrations, binding modes or occlusion size for each of the protein determined. A single binding mode of 54–55 nucleotides was estimated for Alr0088, which was independent of NaCl concentration (Figure 5A). No significant quenching of fluorescence of Alr7579 was observed at low or high concentrations of NaCl (Figure 5B), while two binding modes dependent on NaCl concentration was observed for All4779 (Figure 5C). The binding size was found to be 35.5 nucleotides at 20 mM NaCl and 65.9 nucleotides at 100 mM NaCl for All4779 (Figure 5C), and 32.5 and 70 nucleotides at 20 mM and 100 mM NaCl respectively for EcoSSB under identical experimental conditions, comparable to the $(SSB)_{35}$ and $(SSB)_{65}$ modes of binding, at low and high salt concentrations respectively, shown for EcoSSB [1,27]. Since, $(SSB)_{65}$ mode of binding requires the binding of ssDNA to the tetrameric form of SSB [27], and molecular form corresponding to a tetramer of All4779 was very low, the quenching of fluorescence of All4779 at higher NaCl was lower than that at lower NaCl (Figure 5C), as well as that observed with EcoSSB (Figure 5D).

Thus, though all the three proteins i.e. Alr0088, Alr7579 and All4779 bind ssDNA, their binding affinity and modes of binding are distinct and among these, the binding ability as well as binding modes of All4779 was quite similar to other known bacterial SSBs. The presence of (P/G)-rich spacer and a near identical C-terminal acidic tail, suggests that in *Anabaena* 7120, All4779 may also be performing *in vivo* functions similar to those carried out by the typical bacterial SSB proteins. Since, overexpression of bacterial SSBs are known to influence the repair of stress induced DNA damage [28,29], thereby enhancing tolerance to DNA damaging stresses, a similar role for All4779 was assessed in *Anabaena* 7120.

Physiological Role of All4779 Protein in *Anabaena* 7120

The All4779 protein was overexpressed *in trans* from the plasmid pAM*all4779* (Table 2, Figure 6A) in the recombinant *Anabaena* strain, An*all4779*$^+$ (Table 3). Due to growth under continuous illumination, the expression of the All4779 protein from the light-inducible *psb*A1 promoter was expected to be constitutive. Co-overexpression of the Green Fluorescent Protein (GFP), coded by *gfpmut2* in the pAM*all4779* plasmid, provided a handy tool to distinguish the fully segregated recombinant An*all4779*$^+$ strain exhibiting green fluorescence, from the wild type *Anabaena* 7120 which exhibited red fluorescence upon excitation with λ_{470} light (Figure 6B). It also ensured expression of the upstream gene. The overexpression of All4779 in An*all4779*$^+$ cells was indeed confirmed by immunodetection with anti-All4779 antibody (Figure 6C). Under normal growth conditions, the nitrogen-fixing cultures of An*all4779*$^+$ grew marginally slower than the wild type *Anabaena* 7120 cultures, and at rates comparable to the recombinant *Anabaena* strain harbouring pAM1956 vector, AnpAM (Table 3) [30] (Figure 5D). This is possibly due to the presence of neomycin in the growth medium used for recombinant strain.

The effect of overexpression of All4779 on the ability of *Anabaena* 7120 to tolerate DNA damage inducing stresses was analysed in response to two distinct types of DNA damages i.e. (i) γ-irradiation and desiccation which cause single strand and double strand breaks, and (ii) UV-B and mitomycinC which cause formation of pyrimidine dimers and DNA adducts respectively. The empty vector control recombinant strain, AnpAM exhibited about 55% and 44% survival upon exposure to 6 kGy of ^{60}Co γ-rays or 6 days of desiccation respectively (Figure 7A). Upon constitutive overexpression of All4779 in An*all4779*$^+$ cells, the survival increased to about 60% after exposure to 6 kGy of γ-rays and 70% after 6 days of desiccation (Figure 7A). The recovery of irradiated cultures of *Anabaena*, measured in terms of chlorophyll *a* content increased from about 50% to over 100% in cells overexpressing All4779 (Figure 7B) suggesting better tolerance to radiation. Such correlation was however, not found in post desiccation recovery, (Figure 7B), possibly due to additional stresses, such as osmotic stress experienced during desiccation followed by rehydration of these cells. Of the other two SSB-like proteins of *Anabaena*, overexpression of Alr0088 decreased the radiation tolerance of *Anabaena*, while that of Alr7579 had no effect [14]. This suggested that All4779 is the typical bacterial SSB of *Anabaena*, involved in the repair of single and double strand breaks in DNA, possibly as part of a larger DNA repair complex, which is yet to be identified.

Overexpression of All4779 was also beneficial in protection against stresses which caused formation of DNA adducts. The survival of AnpAM was about 75% and 11.8% respectively upon exposure to 0.75 and 1.5 kJ m^{-2} of UV-B irradiation, which increased to 77% and 50% respectively in An*all4779*$^+$ cells, overexpressing All4779 protein (Figure 7C). The beneficial effect of the constitutive overexpression of All4779 was more pro-

nounced when exposed to higher doses (1.5 kJ m^{-2}) of UV-B (Figure 7C), while at lower dose of (0.75 kJ m^{-2}), that of Alr0088 was more benficial [14]. AnpAM cells exhibited 50% survival upon exposure to 4 μg mitomycinC mL^{-1} for 30 min, which increased to 85% upon overexpression of All4779 in An*all4779*$^{+}$ cells (Figure 7D), comparable to that observed upon overexpression of Alr7579, but lower than that with Alr0088 [14]. Thus, the presence of high levels of All4779 in *Anabaena* possibly decreased the net damage to DNA, both in terms of single and double stranded breaks as well as formation of DNA adducts, possibly by efficient repair of the damaged DNA. Overexpression of SSB has been shown to be beneficial by aiding DNA repair in *E. coli* cells [28].

Thus, All4779 is the major typical bacterial SSB of *Anabaena* 7120 in terms of structural domains, binding to ssDNA and physiological role in DNA repair. The genes coding for the two atypical truncated annotated SSB proteins, Alr0088 and Alr7579 may have arisen due to gene duplication as suggested for PriB, a dimeric protein with only OB-fold and capable of binding ssDNA [31] and may be involved in other functions such as replication and recombination. The unicellular cyanobacterium, *Synechocystis* PCC6803 has been shown to be naturally transformable with possible involvement of competence proteins, ComA (Slr0197) [32] and ComF (Slr0388) [33]. The orthologs of these genes are also found in *Anabaena* 7120, annotated as *all3087* and *alr2926* respectively (http://genome.microbedb.jp/cyanobase/Anabaena), suggesting the possibility of *Anabaena* being also naturally transformable, though this needs to be ascertained. Thus, as has been observed in case of the naturally transformable *B. Subtilis*, the naturally C-terminal truncated BsSsbB, is involved in competence by protecting the incoming DNA [10,11], a similar role may also be associated with Alr0088 or/and Alr7579, both of which bear moderate homology to BsSsb, though this needs to be ascertained. The acidic tail, characteristic of most SSBs, has been shown to be the site of interaction with DNA repair proteins for *E. coli* SSB [3]. Owing to the presence of an acidic tail, All4779 upon overexpression offers better protection from DNA-damage when subjected to different DNA-damage-inducing stresses. Based on data presented, we propose that All4779 be re-annotated as the gene coding for typical single stranded DNA binding protein (SSB) and the corresponding ORF be annotated as the *ssb* gene of *Anabaena* 7120.

Author Contributions

Conceived and designed the experiments: AK HR. Performed the experiments: AK. Analyzed the data: AK HR. Contributed reagents/materials/analysis tools: AK HR. Wrote the paper: AK HR SKA.

References

1. Lohman TM, Ferrari ME (1994) *Escherichia coli* single-stranded DNA-binding protein: multiple DNA-binding modes and cooperativities. Annu. Rev. Biochem. 63: 527–570.
2. Carlini L, Curth U, Kindler B, Urbanke C, Porter RD (1998) Identification of amino acids stabilizing the tetramerization of the single stranded DNA binding protein from *Escherichia coli*. FEBS Lett. 430: 197–200.
3. Shereda RD, Kozlov AG, Lohman TM, Cox MM, Keck JL (2008) SSB as an organizer/mobilizer of genome maintenance complexes. Crit. Rev. Biochem. Mol. Biol. 43: 289–318.
4. Kozlov AG, Cox MM, Lohman TM (2010) Regulation of single-stranded DNA binding by the C termini of *Escherichia coli* single-stranded DNA binding (SSB) protein. J. Biol. Chem. 285: 17246–17252.
5. Eggington JM, Haruta N, Wood EA, Cox MM (2004) The single-stranded DNA- binding protein of *Deinococcus radiodurans*. BMC Microbiol. 4: 2doi 10.1186/1471-2180-4-2.
6. Apte SK (2001) Coping with salinity/water stress: Cyanobacteria show the way. Proc. Indian Natl. Acad. Sci (PINSA), B67: 285–310.
7. Singh H, Fernandes T, Apte SK (2010) Unusual radioresistance of nitrogen-fixing cultures of *Anabaena* strains. J. Biosci. 35: 427–434.
8. Singh H, Anurag K, Apte SK (2013) High radiation and desiccation tolerance of nitrogen-fixing cultures of the cyanobacterium *Anabaena* sp. strain PCC7120 emanates from genome/proteome repair capabilities. Photosynth. Res. 118: 71–81.
9. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK et al. (2011) CDD: a Conserved Domain Database for the functional annotation of proteins. Nucleic Acids Res. 39: 225–229.
10. Yadav T, Carrasco B, Myers AR, George NP, Keck JL, et al., (2012) Genetic recombination in *Bacillus subtilis*: a division of labor between two single-strand DNA-binding proteins. Nuc. Acid Res. 40: 5546–5559.
11. Kidane D, Ayora S, Sweasy JB, Graumann PL, Alonso JC (2012) The cell pole: the site of cross talk between the DNA uptake and genetic recombination machinery. Crit. Rev. Biochem. Mol. Biol. 47: 531–555.
12. Castenholz RW (1988) Culturing methods for cyanobacteria. Methods Enzymol. 167: 68–93.
13. Mackinney G (1941) Absorption of light by chlorophyll solutions. J. Biol. Chem. 140: 315–322.
14. Kirti A, Rajaram H, Apte SK (2013) Characterization of two naturally truncated, Ssb-like proteins from the nitrogen-fixing cyanobacterium, *Anabaena* sp. PCC7120. Photosynth. Res. 118: 147–154.
15. Wadsworth RI, White MF (2001) Identification and properties of the crenarchaeal single-stranded DNA binding protein from *Sulfolobus solfataricus*. Nucleic Acids Res. 29: 914–920.
16. Molineux IJ, Pauli A, Gefter ML (1975) Physical studies of the interaction between the *Escherichia coli* DNA binding protein and nucleic acids. Nucleic Acids Res 2: 1821–1837.
17. Lohman TM, Overman LB (1985) Two binding modes in *Escherichia coli* single strand binding protein-single stranded DNA complexes. Modulation by NaCl concentration. J Biol. Chem. 260: 3594–3603.
18. Chaurasia AK, Parasnis A, Apte SK (2008) An integrative expression vector for strain improvement and environment applications of nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC7120. J. Microbiol. Methods. 73: 133–141.
19. Yoon HS, Golden JW (1998) Heterocyst pattern formation controlled by a diffusible peptide. Science. 282: 935–938.
20. Raghavan PS, Rajaram H, Apte SK (2011) Nitrogen status dependent oxidative stress tolerance conferred by overexpression of MnSOD and FeSOD proteins in *Anabaena* sp. strain PCC7120. Plant Mol. Biol. 77: 407–417.
21. Olszewski M, Grot A, Wojciechowski M, Nowak M, Mickiewicz M, et al. (2010) Charcterization of exceptionally thermostable single-stranded DNA-binding proteins from *Thermotoga maritima* and *Theromotoga neapolitana*. BMC Microbiol. 10: 260. doi.10.1186/1471-2180-10-260.
22. Curth U, Urbanke C, Greipel J, Gerberding H, Tiranti V, et al. (1994) Single-stranded-DNA binding proteins from human mitochondria and *Escherichia coli* have analogous physicochemical properties. Eur. J. Biochem. 221: 435–443.
23. Belts MJ, Russell RB (2003) Amino acid properties and consequences of substitutions. In Bioinformatics for Geneticists. Ed. Barnes MR and Gray IC. John Wiley and sons, 289–316.
24. Bernstein DA, Eggington JM, Killoran MP, Misic AM, Cox MM, et al. (2004) Crystal structure of the *Deinococcus radiodurans* single-stranded DNA-binding protein suggests a mechanism for coping with DNA damage. Proc. Natl. Acad. Sci. 101: 8575–8580.
25. 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: 2706–2711.
26. Reckmann B, Grosse F, Urbanke C, Frank R, Blocker H, et al. (1985) Analysis of secondary structures in M13mp8 (+) single-stranded DNA by the pausing of DNA Polymerase α. Eur. J. Biochem. 152: 633–643.
27. Bujalowski W, Lohman TM (1986) *Escherichia coli* single-strand binding protein forms multiple, distinct complexes with single-stranded DNA. Biochem. 25: 7799–7802.
28. Moreau PL (1987) Effects of overproduction of single-stranded DNA-binding protein on RecA protein-dependent processes in *Escherichia coli*. J. Mol. Biol. 194: 621–634.
29. Moreau PL (1988) Overproduction of single-stranded-DNA-binding protein specifically inhibits recombination of UV-irradiated bacteriophage DNA in *Escherichia coli*. J. Bacteriol. 170: 2493–2500.
30. Rajaram H, Apte SK (2010) Differential regulation of *groESL* operon expression in response to heat and light in *Anabaena*. Arch. Microbiol. 192: 729–738.
31. Ponomarev VA, Makarova KS, Aravind L, Koonin EV (2003) Gene duplication with displacement and rearrangement: origin of the bacterial replication protein PriB from the single-stranded DNA-binding protein, Ssb. J. Mol. Microbiol. Biotechnol. 5: 225–229.
32. Yura K, Toh H, Go M (1999) Putative mechanism of natural transformation as deduced form genome data. DNA Res. 6: 75–82.
33. Nakasugi K, Svenson CJ, Neilan BA (2006) The competence gene, *comF*, from *Synechocystis* sp. strain PCC6803 is involved in natural transformation, phototactic motility and piliation. Microbiol. 152:3623–3631.

Annexures

Nostoc sp. PCC 7120 Ssb1 (ssb1) gene, complete cds (Annexure I)

GenBank: GU225949.1

FASTA Graphics

Go to:

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            Bacteria; Cyanobacteria; Nostocales; Nostocaceae; Nostoc.
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  AUTHORS   Kirti,A., Rajaram,H. and Apte,S.K.
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            and 'Alr7579' Proteins, Is the Major Typical Single-Stranded DNA
            Binding Protein of the Cyanobacterium, Anabaena sp. PCC7120
  JOURNAL   PLoS ONE 9 (4), E93592 (2014)
   PUBMED   24705540
  REMARK    Publication Status: Online-Only
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Nostoc sp. PCC 7120 Ssb2 (ssb2) gene, complete cds (Annexure II)

GenBank: GU225950.1

FASTA Graphics

Go to:

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  ORGANISM  Nostoc sp. PCC 7120
            Bacteria; Cyanobacteria; Nostocales; Nostocaceae; Nostoc.
REFERENCE   1  (bases 1 to 384)
  AUTHORS   Kirti,A., Rajaram,H. and Apte,S.K.
  TITLE     The Hypothetical Protein 'All4779', and Not the Annotated 'Alr0088'
            and 'Alr7579' Proteins, Is the Major Typical Single-Stranded DNA
            Binding Protein of the Cyanobacterium, Anabaena sp. PCC7120
  JOURNAL   PLoS ONE 9 (4), E93592 (2014)
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                     GSPRKEESTSTSAPNETQAVANANF"
     misc_feature    19..306
                     /gene="ssb2"
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/note="SSB_OBF: A subfamily of OB folds similar to the OB fold of ssDNA-binding protein (SSB). SSBs bind with high affinity to ssDNA. They bind to and protect ssDNA intermediates during DNA metabolic pathways. All bacterial and eukaryotic SSBs studied to date...; Region: SSB_OBF; cd04496"
/db_xref="CDD:239942"

misc_feature 10..381
/gene="ssb2"
/note="Single-stranded DNA-binding protein [DNA replication, recombination, and repair]; Region: Ssb; COG0629"
/db_xref="CDD:223702"

misc_feature order(34..42,58..60,88..90,109..114,127..132,136..138, 142..144,148..150,154..156,193..198,220..222,226..228, 232..237,277..279,283..285)
/gene="ssb2"
/note="ssDNA binding site [nucleotide binding]; other site"
/db_xref="CDD:239942"

misc_feature order(19..30,103..111,133..135,139..141,202..204,229..231, 265..267,274..276)
/gene="ssb2"
/note="dimer interface [polypeptide binding]; other site"
/db_xref="CDD:239942"

misc_feature order(202..204,208..210,214..216,298..300)
/gene="ssb2"
/note="tetramer (dimer of dimers) interface [polypeptide binding]; other site"
/db_xref="CDD:239942"

ORIGIN

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       61 tttttcgagt cgggtgcagt taaggcttca atctctattg cagtaagacc gccctacaga
      121 agtgaacaag ccctttggtt tgatttagtc gcttggggaa atcaagcaga agtgattgga
      181 aattacgttc gtaaaggaac tcagattgca attactggtg agtttggatt tgatcgttgg
      241 gctgataaaa attctggcac tatgcgacaa aaacctgtaa tcacaatcaa caccatcgaa
      301 ttattaggtt caccccgtaa agaagaatct acatccactt ctgcaccaaa cgaaacacag
      361 gctgtagcta acgcaaattt ctag
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Nostoc sp. PCC 7120 Ssb3 (ssb3) gene, complete cds (Annexure III)

GenBank: GU225951.1

FASTA Graphics

Go to:

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LOCUS       GU225951                549 bp    DNA     linear   BCT 09-APR-2014
DEFINITION  Nostoc sp. PCC 7120 Ssb3 (ssb3) gene, complete cds.
ACCESSION   GU225951
VERSION     GU225951.1  GI:281398188
SOURCE      Nostoc sp. PCC 7120 (Anabaena sp. PCC 7120)
  ORGANISM  Nostoc sp. PCC 7120
            Bacteria; Cyanobacteria; Nostocales; Nostocaceae; Nostoc.
REFERENCE   1  (bases 1 to 549)
  AUTHORS   Kirti,A., Rajaram,H. and Apte,S.K.
  TITLE     The Hypothetical Protein 'All4779', and Not the Annotated 'Alr0088'
            and 'Alr7579' Proteins, Is the Major Typical Single-Stranded DNA
            Binding Protein of the Cyanobacterium, Anabaena sp. PCC7120
  JOURNAL   PLoS ONE 9 (4), E93592 (2014)
   PUBMED   24705540
FEATURES             Location/Qualifiers
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                     /organism="Nostoc sp. PCC 7120"
                     /mol_type="genomic DNA"
                     /strain="PCC 7120"
                     /db_xref="taxon:103690"
     gene            1..549
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     CDS             1..549
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                     /note="single stranded DNA binding protein"
                     /codon_start=1
                     /transl_table=11
                     /product="Ssb3"
                     /protein_id="ADA67920.1"
                     /db_xref="GI:281398189"
                     /translation="MNSCVLLAEIIQEPQLRYTSDNLAVTEMLVQFPNSQKPDDPPAT
                     LKVVGWGNLATEIQQNYHQGDRVIIAGRLGMNTIDRPEGFKEKRAELTVQQIQSISGS
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                     YEPAPQTTYSPVEVPDPDDIPF"
     misc_feature    10..297
                     /gene="ssb3"
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/note="SSB_OBF: A subfamily of OB folds similar to the OB fold of ssDNA-binding protein (SSB). SSBs bind with high affinity to ssDNA. They bind to and protect ssDNA intermediates during DNA metabolic pathways. All bacterial and eukaryotic SSBs studied to date...; Region: SSB_OBF; cd04496"
/db_xref="CDD:239942"

misc_feature 1..546
/gene="ssb3"
/note="Single-stranded DNA-binding protein [DNA replication, recombination, and repair]; Region: Ssb; COG0629"
/db_xref="CDD:223702"

misc_feature order(25..33,49..51,79..81,100..105,121..126,130..132,136..138,142..144,148..150,187..192,214..216,220..222,226..231,268..270,274..276)
/gene="ssb3"
/note="ssDNA binding site [nucleotide binding]; other site"
/db_xref="CDD:239942"

misc_feature order(10..21,94..102,127..129,133..135,196..198,223..225,256..258,265..267)
/gene="ssb3"
/note="dimer interface [polypeptide binding]; other site"
/db_xref="CDD:239942"

misc_feature order(196..198,202..204,208..210,289..291)
/gene="ssb3"
/note="tetramer (dimer of dimers) interface [polypeptide binding]; other site"
/db_xref="CDD:239942"

ORIGIN

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        1 atgaacagct gtgttttatt agcagaaatc attcaagagc cacaactgcg ctatacatct
       61 gataatttag cagtcacaga aatgttggtg caatttccca attcccaaaa accagatgat
      121 ccaccagcga ctttaaaagt tgttggctgg gggaatttag ctacagaaat ccagcaaaac
      181 tatcatcaag gcgatcgcgt catcattgct ggacgtttag ggatgaatac catcgaccgt
      241 ccagaaggat ttaaagaaaa acgggctgaa ttaacagtac agcagattca atcaattagt
      301 ggtagtggta acttcacctc atcaccagca gtatccagaa caccagccgt taccgaaacc
      361 ccctctcgtc caccctcccc ggctgttacg actcaacatg atcttcctag ctacgaatca
      421 ccacgtccag caccaactcc agcacccagt aaagtgagtg ttccacccca ggtaaacaac
      481 tatgaacctg cgcctcaaac tacctactca cctgtagaag taccagaccc agacgatatt
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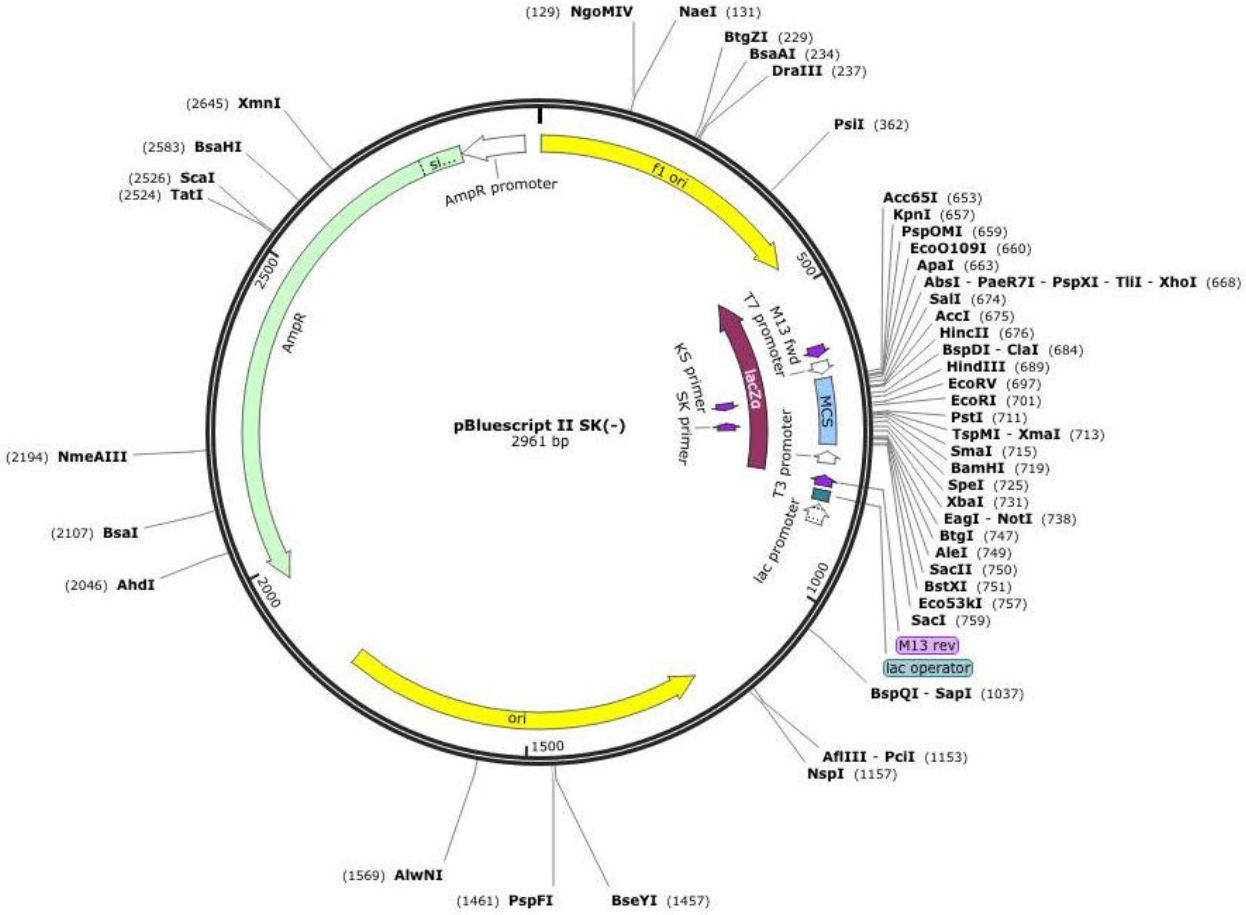
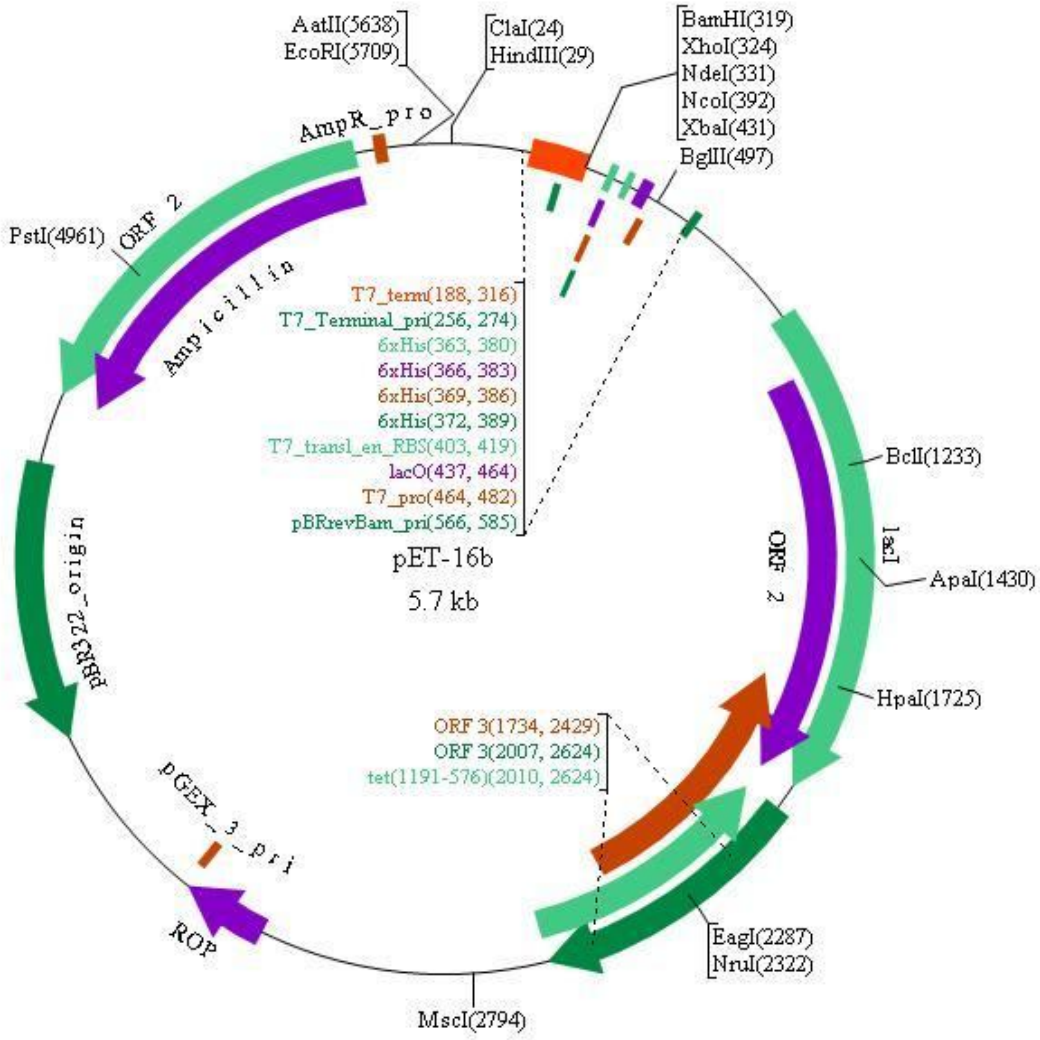
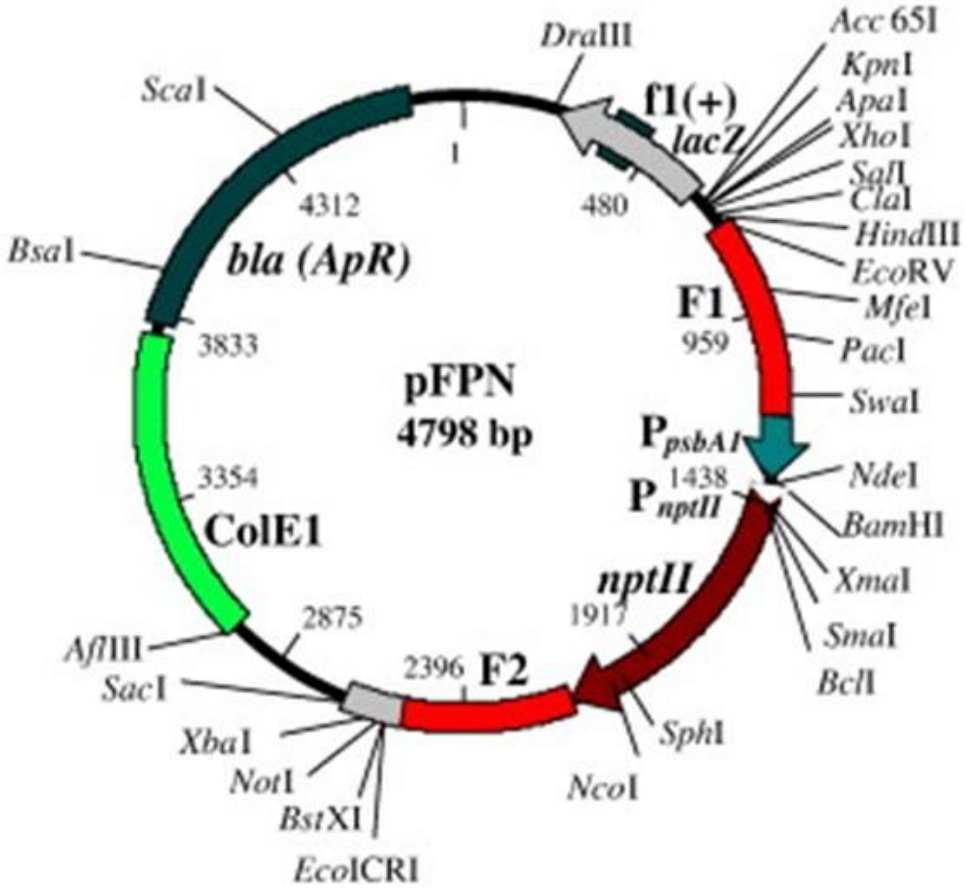
pBlueScript,pBS SK (-) vector map (Annexure IV)



pET16b vector map (Annexure V)



pFPN vector map (Annexure VI)



pAM1956 vector map (Annexure VII)