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Functional roles of N-terminal and C-terminal domains in the overall activity of a novel single-stranded DNA binding protein of *Deinococcus radiodurans*

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ABSTRACT

Single-stranded DNA binding protein (Ssb) of *Deinococcus radiodurans* comprises N- and C-terminal oligonucleotide/oligosaccharide binding (OB) folds connected by a beta hairpin connector. To assign functional roles to the individual OB folds, we generated three Ssb variants: Ssb_N (N-terminal without connector), Ssb_{NC} (N-terminal with connector) and Ssb_C (C-terminal), each harboring one OB fold. Both Ssb_N and Ssb_{NC} displayed weak single-stranded DNA (ssDNA) binding activity, compared to the full-length Ssb (Ssb_{FL}). The level of ssDNA binding activity displayed by Ssb_C was intermediate between Ssb_{FL} and Ssb_N. Ssb_C and Ssb_{FL} predominantly existed as homo-dimers while Ssb_{NC}/Ssb_N formed different oligomeric forms. *In vitro*, Ssb_{NC} or Ssb_N formed a binary complex with Ssb_C that displayed enhanced ssDNA binding activity. Unlike Ssb_{FL}, Ssb variants were able to differentially modulate topoisomerase-I activity, but failed to stimulate Deinococcal RecA-promoted DNA strand exchange. The results suggest that the C-terminal OB fold is primarily responsible for ssDNA binding. The N-terminal OB fold binds weakly to ssDNA but is involved in multimerization.

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1. Introduction

Deinococcus radiodurans exhibits extreme resistance to radiation, desiccation and chemical mutagens [1–3]. This resistance is conferred by a variety of coping strategies adopted by the bacterial cell, such as high manganese to iron ratios, Extended Synthesis Dependent Strand Annealing (ESDSA), a condensed nucleoid structure and robust error-free DNA repair system [4–6]. In bacteria, the single-stranded DNA binding protein (Ssb) is crucial for all aspects of DNA metabolism [7]. It harbours Oligonucleotide/Oligosaccharide Binding (OB) folds which bind to ssDNA by electrostatic and base stacking interactions [8–10]. Ssb is required for DNA replication and repair processes where it keeps DNA in single-stranded form and protects it from nucleolytic degradation [11]. Ssb protein is also known to modulate the activities of several enzymes/proteins (DNA polymerases, primases, RecQ, RecO, RNA polymerases, etc.) either by direct interaction with the enzymes *via* its C-terminal acidic tail or indirectly by modulating the topology of DNA by removing secondary structure or by keeping it in single-stranded form [7,12–15].

Deinococcus-Thermus group of bacteria encode Ssb proteins which are different from that of the prototype *Escherichia coli* Ssb. They contain two OB folds and function as homodimers [16], in contrast to the homotetrameric *E. coli* Ssb that harbours a single OB fold or heterotrimeric eukaryotic RPA (Replication protein A) [12,17]. In *D. radiodurans*, Ssb is known to be transcriptionally induced following radiation and mitomycin-C exposure and its expression levels are controlled by two radiation and desiccation response motifs found upstream of the *ssb* promoter [18]. Transcriptomic and proteomic analyses of Deinococcal cells following gamma radiation stress have revealed up-regulation of repair proteins [19,20]. Among these, Ssb has been shown to be the most abundant protein [21], which together with gyrase, RecA, topoisomerase I and RecQ helicase, is recruited to the Deinococcal nucleoid [22]. The lethality of a Ssb deletion cannot be complemented by DdrB, which is the other ssDNA binding protein found in Deinococcal species [23]. Biochemical analyses have revealed

Abbreviations: ESDSA, extended synthesis-dependent strand annealing; OB fold, oligonucleotide/oligosaccharide binding fold; RPA, Replication protein A; Ssb, single-stranded DNA binding protein; Ssb_C, C-terminal Ssb; Ssb_N, N-terminal Ssb without connector; Ssb_{NC}, N-terminal Ssb with connector; ssDNA, single-stranded DNA

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that Deinococcal Ssb displays salt-independent weak ssDNA binding activity and displaces shorter strand of duplex DNA than *E. coli* Ssb [24,25].

Bioinformatic analyses indicated differences between the two OB folds [16], later confirmed by crystal structure, which revealed a structural asymmetry between the two domains and suggested that each OB fold may have evolved for a specialized role. It also showed that Deinococcal Ssb dimer formation occurred using an extensive surface area formed by N-terminal and the connector region. The interface had hydrogen, ionic and van der Waals interaction and was quite different from *E. coli* Ssb tetramer formation which involved L_{45} loop mediated interaction [9,26,27].

In this study, we have performed structure-based functional analysis of *D. radiodurans* Ssb. The nucleotide sequences corresponding to the full length protein (Ssb_{FL}, amino acids 1–301), N-terminal OB domain (Ssb_N, amino acids 1–114), N-terminal OB domain with connector ($Ssb_{NC,}$ amino acids 1–124) and C-terminal OB domain (Ssb_C, amino acids 125–301) were cloned, over-expressed and purified from *E. coli*. Protein–DNA and protein–protein interaction assays revealed differential ssDNA binding and oligomerization by different domains as well as demonstrated physical and functional interaction to enhance ssDNA binding between N-terminal and C-terminal domains. All the variants of Ssb and the combination of protein domains containing one or two OB folds were capable of localized melting of ssDNA and assisted topoisomerase activity but failed to remove secondary structures and stimulate Deinococcal RecA-promoted strand exchange compared to full length Ssb. The data demonstrates specific roles for two individual OB folds of Deinococcal Ssb and their roles in enhancing the efficacy of Deinococcal Ssb.

2. Materials and methods

2.1. Enzymes, buffers and fine biochemicals

Restriction enzymes, vectors (pTWIN1 & pMBX10), DNA (ØX virion, ØX dsDNA and M13mp18 dsDNA), *E. coli* Topoisomerase I and chitin beads were obtained from New England Biolabs, UK. Q-Sepharose, Sephadex 50 columns, terminal transferase, Dig labeled ddUTP, anti-Dig antibody and NBT-BCIP solution were obtained from Roche Life Sciences, UK. Ni–NTA matrix was obtained from Qiagen, Germany. IPTG, ATP, phosphocreatine, phosphocreatine kinase and *E. coli* Ssb protein were obtained from Sigma–Aldrich India. Bacterial growth medium component were obtained from BD and Co., India. Oligo dT50 was obtained from MWG Biotech, India. Genomic DNA isolation kit was obtained from Hi-media Laboratories, India.

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

The oligonucleotide primers, plasmids and recombinant strains are described in Table 1. PCR amplifications were carried out from *D. radiodurans* (R1) genomic DNA as previously described [18]. The full length *ssb* gene (Ssb_{FL}) was amplified using specific primers FLF and FLR (Table 1) digested with *Bam*HI and ligated to pUC19 at *Bam*HI site to obtain plasmid pUC19Ssb_{FL}. The insert from pUC19Ssb_{FL} was released by digesting it with *Nde*I and *Bam*HI and then ligating it to pET16b at corresponding sites to generate plasmid pETSsb_{FL}. Ssb-C-terminal domain (Ssb_C) was PCR amplified by using primers CTF and CTR and cloned at *Nde*/*Bam*HI restriction sites of pET16b to obtain plasmid pETSsb_C. Ssb-N-terminal with connector was amplified by using primers NCF and NCD,

Table 1
Strains, plasmids and primers used in this study.

Host cells, plasmid or primer	Description	Reference, source or remarks
Bacterial strains		
Deinococcus radiodurans	*Deinococcus radiodurans* R1, ATCC BAA-816	Lab collection
E. coli DH5α	F^- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169, hsdR17(r_K^- m_K^+), λ–	Lab collection
E. coli BL-21pLysS	F^- ompT gal dcm lon hsdS_B(r_B^- m_B^-) λ(DE3) pLysS(cm^R)	Novagen
E. coli ER2566	*fhuA2 lacZ::T7 gene1 [lon] ompT gal sulA11 R(mcr-73::miniTn10*–TetS*)2 [dcm] R(zgb-210::Tn10*– TetS) *endA1 Δ(mcrC-mrr)114::IS10*	NEB
E. coli STL2669	(Δ(recA-srlR)306:Tn10 xonA2(sbcB⁻))	Lab collection
E. coli BLSsb_{FL}	*E. coli* BL-21pLysS cells containing recombinant plasmid pET16b-Ssb_{FL}	This study
E. coli BLSsb_C	*E. coli* BL-21pLysS cells containing recombinant plasmid pET16b-Ssb_C	This study
E. coli ERSsb_{NC}	*E. coli* ER2566 cells containing recombinant plasmid pTWIN1-Ssb_{NC}	This study
E. coli ERSsb_N	*E. coli* ER2566 cells containing recombinant plasmid pMXB10-Ssb_N	This study
E. coli STLRecA	*E. coli* STL2669 cells containing recombinant plasmid pET16b-RecA devoid of any His-Tag	This study
Plasmids		
pET16b	Amp^r, protein expression vector having His-Tag	Novagen
pTWIN1	Amp^r, protein expression vector having intein Tag	NEB
pMXB10	Amp^r, protein expression vector having intein Tag	NEB
pETSsb_{FL}	Deinococcal Ssb_{FL} gene cloned at *Nde*/*Bam*HI restriction sites	This study
pETSsb_C	C-terminal region of Deinococcal Ssb cloned at *Nde*/*Bam*HI	This study
pETRecA	Deinococcal RecA gene cloned at *Nco*I/*Bam*HI restriction sites	This study
pTWINSsb_{NC}	N-terminal with connector region of Deinococcal Ssb cloned at *Eco*RI/*Bam*HI restriction sites	This study
pMXB10Ssb_N	N-terminal region of Deinococcal Ssb cloned at *Nde*I/*Xho*I restriction sites	This study
Primers		
FLF	5-CCAGGGATCCAAGGAGAATTTGTTCATATGG-3	*Bam*HI, *Nde*I
FLR	5-CGCTGTTTCCTTGCTGGATCCTGTTG-3	*Bam*HI
CTF	5-TGATTCAGGATCCTGGCGGCGGCGTGCATATGAGC-3	*Nde*I
CTR	5′GCTGGGTCATGTTGGGGATCCTTGGTG-3′	*Bam*HI
NCF	5-GGAATTCCATATGGCCCGAGGCATGAAC-3	*Eco*RI
NCD	5-CGGGATCCTTAGCGCACGCCGCCGCC-3	*Bam*HI
NF	5-GGAATTCCATATGGCCCGAGGCATGAAC-3	*Nde*I
ND	5-CCGCTCGAGTTCGGGCTGGGTGCC-3	*Xho*I
RF	5-CATGCCATGGCCATGAGCAAGGACGCC-3	*Nco*I
RD	5-CCGCTCGAGGTTTACGCTTCGGCGGC-3	*Xho*I

Restriction site contained in primer is underlined.

subsequently was cloned in pTwin1 vector at *Eco*RI and *Bam*HI sites to generate pTwinSsb$_{NC}$. Ssb-N-terminal region without connector was amplified using primers NF and ND, subsequently was cloned in pMXB10 vector at *Nde*I and *Xho*I sites to construct pMXB10Ssb$_{N}$. *D. radiodurans recA* gene was amplified by using primers RF and RD and cloned in pET16b at *Nco*I (to avoid His tag) and *Xho*I sites to generate pETRecA. The identity of all the clones was ascertained by DNA sequencing.

2.3. Over expression and purification of Ssb$_{FL}$ and Ssb$_{C}$

The constructs pETSsb$_{FL}$ and pETSsb$_{C}$ were transformed separately into *E. coli* BL-21(pLysS) cells to obtain strains BLSsb$_{FL}$ and BLSsb$_{C}$. Cells were grown in LB medium at 37 °C and induced by the addition of IPTG to a final concentration of 1 mM. The His-tagged proteins were purified using Ni–NTA (nickel–nitrilo triacetic acid) resin and eluted with imidazole gradient (1 → 500 mM). Individual proteins were dialyzed overnight to remove imidazole. Trace amounts of protein contaminants were removed by passing the proteins over Q-Sepharose affinity matrix. The homogeneous fractions which were devoid of both endo- and exonucleases were pooled and dialyzed in Ssb storage buffer (20 mM Tris, pH 8.3, 500 mM NaCl, 1 mM EDTA, 1 mM 2-mercaptoethanol and 50% glycerol), snap-frozen in liquid nitrogen and stored at −80 °C.

2.4. Over expression and purification of Ssb$_{NC}$ and Ssb$_{N}$

The pTwinSsb$_{NC}$ and pMXB10Ssb$_{N}$ constructs were separately transformed into *E. coli* strain ER2566 to obtain ERSsb$_{NC}$ and ERSsb$_{N}$. Cells were grown in LB medium at 37 °C and over expressed by adding IPTG to a final concentration of 1 mM at 20 °C for 12 h. The clarified cell free extracts (in 20 mM Tris, pH 8.5, 0.5 M NaCl) were loaded onto a chitin column and washed with 20 volumes of column buffer (20 mM Tris, pH 8.5, 0.5 M NaCl). On-column cleavage of Ssb$_{NC}$ was done by equilibrating the column with 20 mM sodium phosphate buffer pH 6.0 containing 0.5 M NaCl and incubating it at 4 °C for 4 days. For Ssb$_{N}$, column was equilibrated with 20 mM Tris–HCl, 0.5 M NaCl pH 8.5 buffer containing 10 mM DTT (dithiothreitol) and incubated overnight at 4 °C. Bound proteins were eluted by passing equilibration buffer without DTT. The fractions were analyzed by 14% SDS–PAGE. To remove trace amounts of impurities, the eluted fractions of Ssb$_{NC}$ or Ssb$_{N}$ were passed through Q-Sepharose column and eluted by a (20 mM → 1000 mM) NaCl gradient. The fractions free of both endo- and exonucleases were pooled and dialyzed in Ssb storage buffer, snap-frozen in liquid nitrogen and stored at −80 °C.

2.5. Over expression and purification of RecA protein

Deinococcal RecA protein was purified as previously described [28] with some modifications. Briefly, pETRecA construct was transformed into *E. coli* STL2669 cells to obtain strain STLRecA and over expressed by adding IPTG (1 mM final concentration) at 37 °C for 4 h. Cells were lysed by sonication and the crude lysate was centrifuged at 40,000*g* for 1 h. RecA protein was precipitated by adding 10% Polymin-P (pH 7.9) to a final concentration of 0.5% over 15 min with continuous stirring. The pellet was extracted until much of Polymin-P is removed as described [28]. Other trace contaminating proteins were removed by affinity Q-Sepharose column and eluted with gradient (20 mM → 1000 mM) NaCl. The nuclease free protein aliquots were dialyzed overnight in RecA storage buffer (20 mM Tris–HCl, pH 7.5, 1 mM DTT and 10% glycerol) and stored at −80 °C.

2.6. Oligomeric status determination

Oligomeric status of all the Ssb variants was determined by Superdex-75 gel chromatography. Standard graph for the column was prepared based on the elution volume obtained for Bovine serum albumin (66 kDa), Ovalbumin (44 kDa), Carbonic anhydrase (29 kDa) and Cytochrome-C (12.4 kDa) protein standards. Approximately 250 μl of all Ssb variants (∼2.5 mg/ml) were passed through column individually. The flow rate was maintained at 0.5 ml per min. Ssb$_{FL}$ or Ssb$_{C}$ eluted as single major peak (fraction F1). Ssb$_{N}$ or Ssb$_{NC}$ formed two major peaks. First peak (high molecular mass) eluted in the void volume of the column (fractions F1 and F2), while the second peak was collected as fraction F3.

2.7. Electrophoretic Mobility Shift Assays (EMSA)

EMSA was carried out with individual Ssb$_{FL}$, Ssb$_{C}$, Ssb$_{NC}$ or Ssb$_{N}$ and Dig labeled dT50 oligo. The indicated protein was incubated with the dT50 oligo in 20 mM Tris–acetate pH 7.4 and 1 mM Mncl$_2$ at 27 °C for 20 min after which the DNA–protein complexes were resolved on a 12% native PAGE for 5 h at 50 V in 40 mM Tris acetate buffer, pH 7.4. The DNA–protein complexes were electro blotted onto nylon membrane, probed with anti-DIG antibody and developed using NBT-BCIP (nitro-blue tetrazolium and 5-bro mo-4-chloro-3′-indolyphosphate) colorimetric substrate. The bands formed were quantitated by gel quant software (Biosystematica, UK) and data was fitted to Hill's equation to obtain K_D value for Ssb$_{FL}$ and Ssb$_{C}$. Each experiment was repeated three times and K_D values were obtained from each experiment. The K_D values (with standard deviation) depicted, were calculated by taking average of three independent experiments [29]. EMSA as described above was also carried out with different combinations of Deinococcal Ssb variants (Ssb$_{NC}$ or Ssb$_{N}$ and Ssb$_{C}$) and also with combination of Ssb$_{NC}$/Ssb$_{N}$ and *E. coli* Ssb.

2.8. Domain interaction studies

Purified Ssb$_{C}$ was bound to Ni–NTA matrix through its His tag. Ssb$_{NC}$ or Ssb$_{N}$ was then added to Ni–NTA attached Ssb$_{C}$ separately. As a control, Ssb$_{NC}$ or Ssb$_{N}$ was also incubated separately with Ni–NTA matrix to check any non-specific binding. The columns were washed with increasing concentration of imidazole (0 → 200 mM) and final elution was done with 250 mM imidazole. The elutant so obtained were resolved by 14% SDS–PAGE and stained with CBB.

2.9. Stimulation of E. coli topoisomerase activity by Deinococcal Ssb variants

The effect of various Deinococcal Ssb variants on *E. coli* topoisomerase activity was investigated as described [30]. The reaction was carried out in a buffer provided by the supplier (NEB) with 0.5 μg of M13mp18 negatively superhelical (form I) DNA, 0.1 unit topoisomerase I and indicated concentration of Ssb variants at 37 °C for 15 min. The reaction was terminated by the addition of EDTA to a final concentration of 10 mM and further incubated at 65 °C for 20 min. After the addition of SDS (0.8% final concentration), samples were resolved on a 0.8% agarose gel at 23 V in 0.5× TBE buffer (pH 8.2) for 16 h. The gels were subsequently stained with ethidium bromide (1–2 μg/ml) and visualized and photographed under UV using Syngene gel documentation system.

2.10. Strand exchange assay

The assay was performed as described [31–33]. Briefly, the reaction mixtures contained 10 μM Øx174 ssDNA and 10 μM Øx174

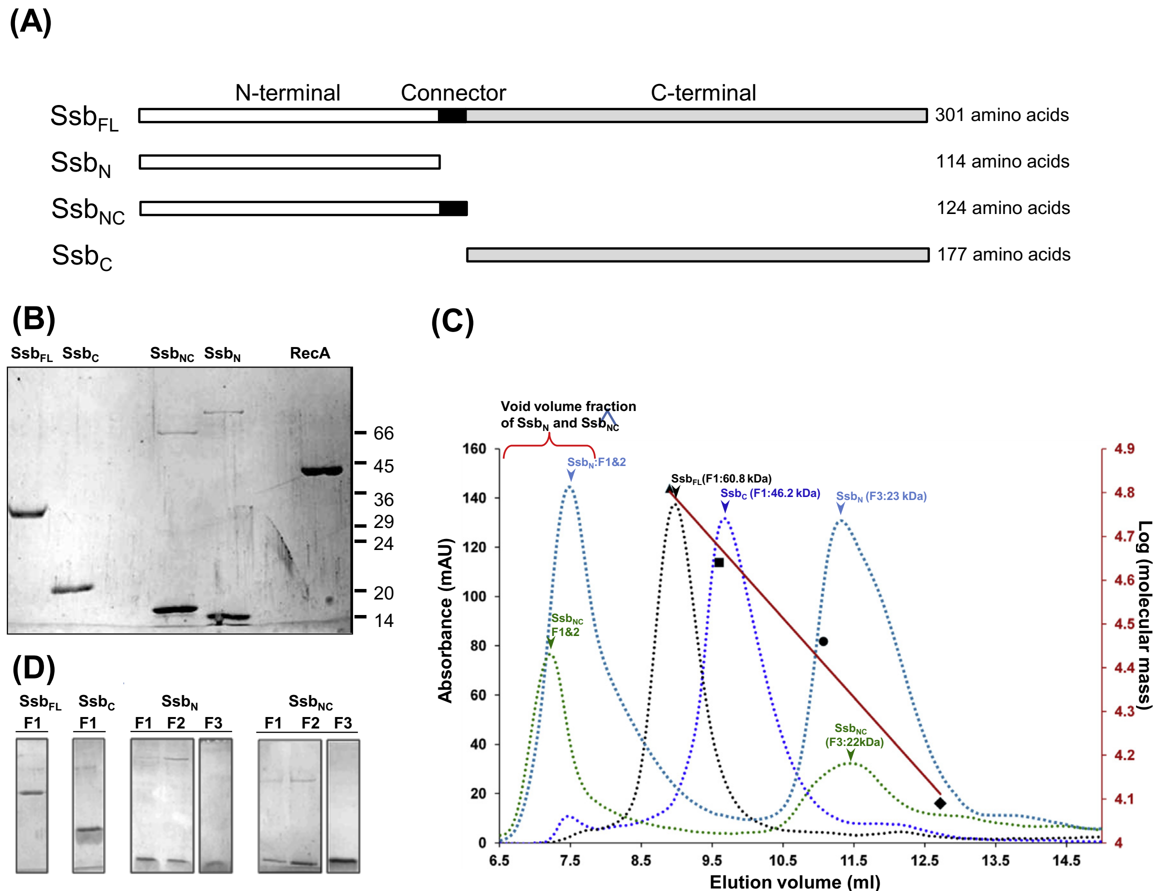

Fig. 1. Purification and solution status of Deinococcal Ssb protein variants. (A) Schematic representation of Deinococcal Ssb protein and its variants. (B) SDS PAGE profiles of purified proteins (1 μg each) of Ssb_{FL}, Ssb_C, Ssb_{NC}, Ssb_N and RecA. (C) Oligomeric status of Ssb variants was determined by gel exclusion chromatography. Elution profile of all the Ssb variants is shown. Standard curve was drawn based upon the elution profile of the following protein standards (▲, Bovine Serum Albumin: 66 kDa; ■, Ovalbumin: 44 kDa; ●, Carbonic anhydrase: 29 kDa; ♦, Cytochrome C: 12.4 kDa). The continuous red line indicates linear fit of standard curve. The calculated molecular mass and fraction number of all Ssb variants is shown. Molecular mass of void volume fractions (F1 and F2) of Ssb_{NC} and Ssb_N could not be determined. (D) Fractions of Ssb variants resolved by 14% SDS PAGE. The fraction number of all Ssb variants is shown.

linear double stranded DNA (dsDNA) in 20 mM Tris acetate pH 7.9, 50 mM potassium acetate, 10 mM magnesium acetate and 1 mM dithiothreitol, ATP (3 mM) and ATP regeneration system (12 mM phosphocreatine and 10 units/ml of phosphocreatine kinase). In one set of reactions (SS–DS mode) Øx174 ssDNA was first incubated with 3 μM Deinococcal RecA in reaction buffer containing 3 mM ATP for 10 min at 37 °C. Individual variants of Ssb proteins or their combination were then added and incubated for another 10 min. Finally, reaction was initiated by addition of linear Øx174 dsDNA. A parallel set of reactions (DS–SS mode) in which RecA was first incubated with linear ØX174 DS DNA for 40 min, then ØX174 ssDNA was added, and after 5 min of incubation Ssb was added [34]. In both, the reaction was terminated after 2 h by adding SDS to a final concentration of 1.25% and the reaction products were resolved on a 0.8% agarose gel at 23 V in 0.5× TBE buffer (pH 8.2) for 16 h. The gel was stained with ethidium bromide and the products were visualized and photographed under UV using Syngene gel documentation system.

3. Results and discussion

3.1. Cloning and purification of Deinococcal Ssb variant forms

Sequence similarity analyses of Deinococcal Ssb showed that Ssb_{NC} shares 33% identity and 51% similarity with Ssb_C and 38% identity and 49% similarity with *E. coli* Ssb (Blastp analysis, data not shown). Ssb_C shared 39% identity and 64% similarity with *E. coli* Ssb protein [16] and is similar to *E. coli* Ssb than to Ssb_{NC}. A schematic of various Deinococcal Ssb variants studied in this work is shown in Fig. 1A. The Deinococcal Ssb variant forms and Deinococcal RecA were cloned, over-expressed in *E. coli* and purified as detailed under Section 2. Ssb_{FL} and Ssb_C were expressed with Histidine Tag. Ssb_{NC} and Ssb_N were expressed with intein tag, which was subsequently removed. The purified proteins (Fig. 1B) were devoid of both exo- and endonuclease activity (data not shown). All these proteins were then subjected to various biochemical analyses.

3.2. Ssb domains display differential oligomerization properties

E. coli Ssb, that harbors a single OB fold, is known to form a tetramer [35]. Oligomeric forms of Deinococcal Ssb variants were separated by gel filtration chromatography. Ssb_{FL} (monomer with His-tag – 35 kDa) was found to exist as a dimer of 60.8 kDa (Fig. 1C). Ssb_C (monomer with His-tag – 21.6 kDa) showed similarity to *E. coli* Ssb at the amino acid level, but formed a dimer of 46.2 kDa (Fig. 1C). Ssb_{NC} and Ssb_N proteins displayed polydispersivity. Ssb_{NC} (monomer 14.7 kDa with additional 10 amino acids) formed a dimer (22 kDa) as well as complex multimers of high molecular mass which eluted in the void volume of the column. Ssb_N (monomer 13 kDa with additional 3 amino acids) also existed in diverse oligomeric forms. The dimeric form (23 kDa) and

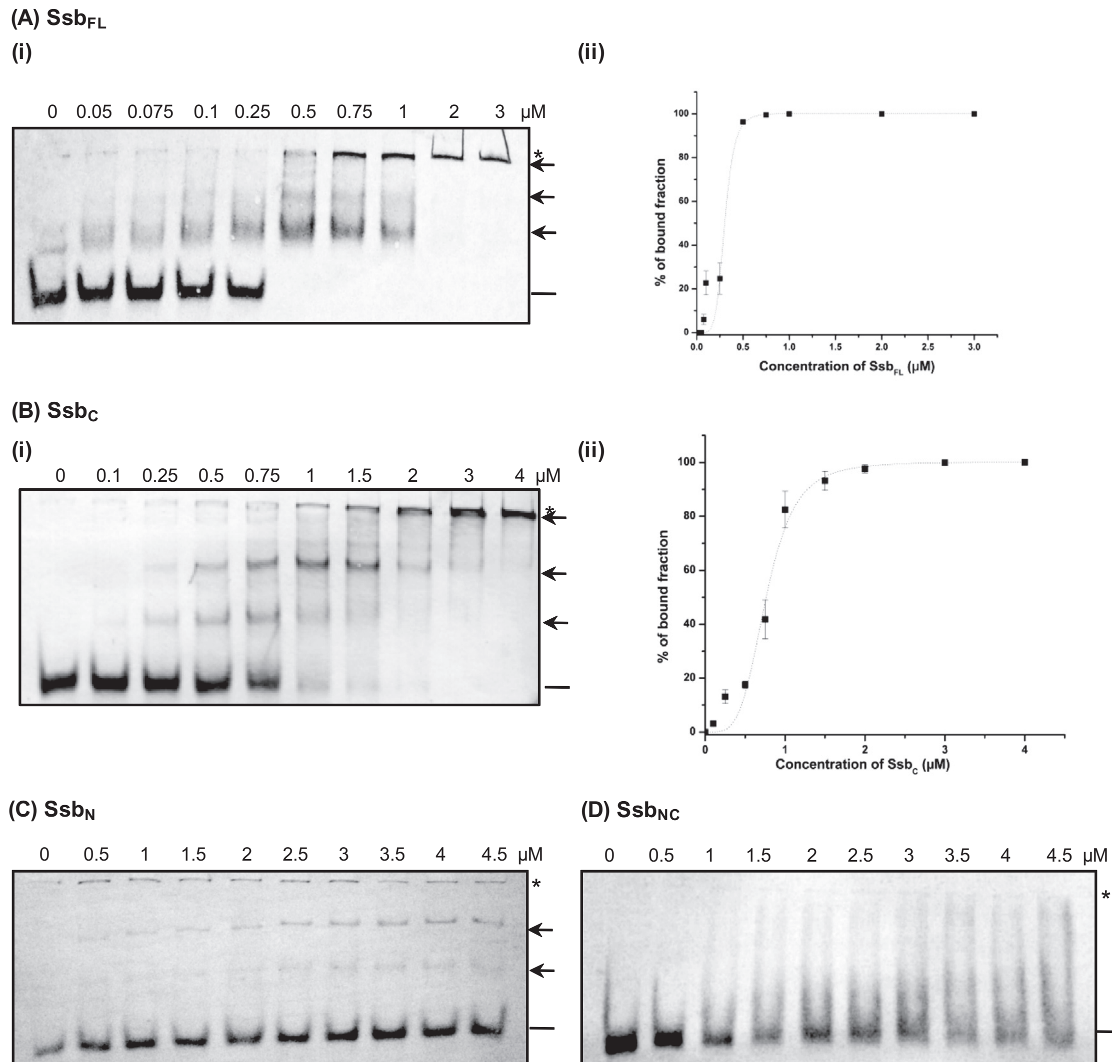

Fig. 2. Single-stranded DNA binding activity of Deinococcal Ssb protein variants. The indicated concentrations of Ssb protein variants were incubated with oligo dT (0.125 µM) for 20 min at 27 °C and resolved by 12% native PAGE. The amount of protein–DNA complexes was quantified for Ssb_{FL} and Ssb_C using Gel Quant software. The representative graph for Ssb_{FL} [panel A (ii)] and Ssb_C [panel B (ii)] is shown. The data points were fitted into Hill's equation (dotted line) to determine the K_D values. The error bars represent standard deviation of three independent experiments. DNA substrate and Ssb-protein complex are shown by "—" and "←", respectively while wells of the gels are marked by asterisk. (A) Ssb_{FL} (B) Ssb_C (C) Ssb_N (D) Ssb_{NC}.

complex multimeric forms of high molecular mass were clearly visualized as discrete peaks in the elution profile (Fig. 1C) or as distinct bands (Fig. 1D). Differences between the observed and theoretical molecular weights of Ssb_{NC} noted in this study has also been reported earlier [36], which may be attributable to the presence of hydrophobic amino acid patches. The shoulder seen in Ssb_N dimeric profile could be contributed by small fraction of monomeric protein of Ssb_N, which is co-eluting in the same peak profile. The oligomerization pattern of Ssb variants clearly demonstrated that the multimerization property resided with the N-terminal domain of Ssb and may help in stabilization of dimeric state of Ssb_{FL}.

3.3. Ssb domains exhibit differential ssDNA binding

The ssDNA binding activity of Deinococcal Ssb variants was tested by Electrophoretic Mobility Shift Assay (EMSA) using oligo dT50 over a range of protein concentrations (Fig. 2). Among the various Ssb proteins, Ssb_{FL} displayed highest ssDNA binding activity [Fig. 2A(i)] followed by Ssb_C [Fig. 2B(i)]. The apparent equilibrium dissociation constant (K_D) was calculated to be 0.76 ± 0.04 µM for Ssb_C (Hill coefficient: 4.25 ± 0.48) as compared to 0.30 ± 0.01 µM for Ssb_{FL} (Hill coefficient: 5.63 ± 1.06), indicating that the ssDNA binding affinity of Ssb_C was lower than Ssb_{FL}. The Hill coefficients obtained were in agreement to those obtained earlier for other DNA binding proteins including human Ssb protein [37,38]. In comparison, Ssb_N and Ssb_{NC} bound ssDNA weakly but gave different patterns of DNA–protein complexes [compare Fig. 2(C) with Fig. 2(D)]. Ssb_{NC} displayed a smear-like pattern and a distinct decrease in target DNA with increasing protein concentration [Fig. 2(D)], whereas Ssb_N showed discrete bands of DNA–protein complex but with no significant decrease in target DNA. Cooperative ssDNA binding by Ssb_{FL} has been demonstrated earlier using long ssDNA molecules of M13 bacteriophage [24]. Among all the Ssb variants, Ssb_{FL} had highest affinity for ssDNA, followed by Ssb_C while the N-terminal region had the least. The presence of connector region appeared to improve ssDNA binding of the N-terminus.

3.4. Ssb_N/Ssb_{NC} and Ssb_C display physical and functional interaction in vitro

The functional and physical interactions were determined by EMSA and affinity chromatography. To test direct interaction

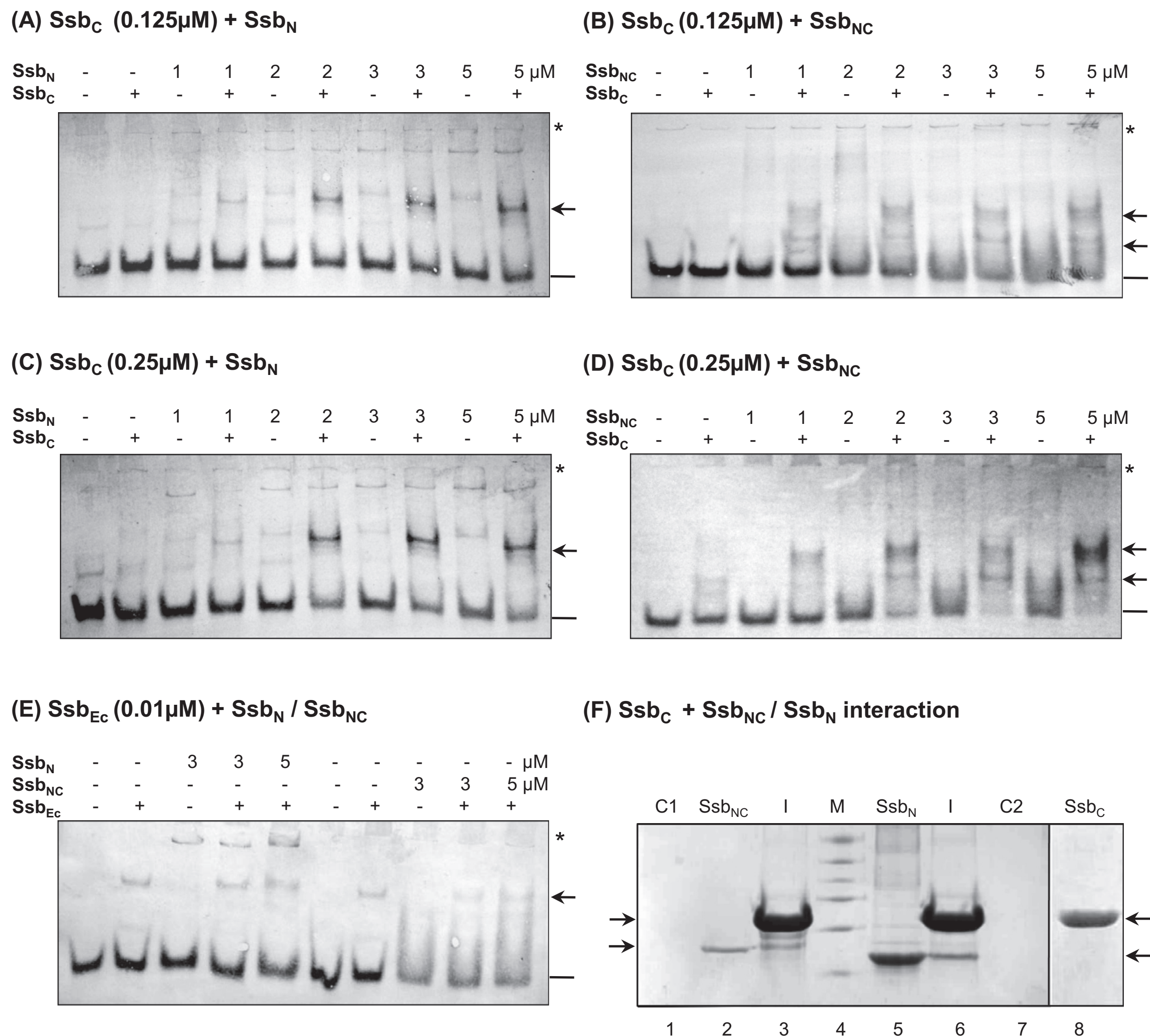

Fig. 3. *In vitro* functional interactions of Deinococcal $Ssb_{N/NC}$ with Ssb_C. (A,B) EMSA was carried out as detailed in Fig. 2 except that indicated concentration of Ssb_N (A) and Ssb_{NC} (B) were mixed with fixed concentration of Ssb_C (0.125 μM) and then incubated with 0.125 μM oligo dT50. The presence or absence of Ssb_C protein is indicated by "+" or "-" sign above each lane. (C,D) EMSA was carried out as detailed in (A, B) except that concentration of Ssb_C was fixed at 0.25 μM. (E) EMSA was carried out as detailed in (A) except that Ssb_C was replaced by 0.01 μM *E. coli* Ssb. DNA substrate and Ssb-protein complex are shown by "—" and "←", respectively while wells of the gels are marked by asterisk. (F) Interaction of individual Deinococcal Ssb domains. Ssb_C (bait) was bound to Ni–NTA by its His-tag and the column was equilibrated with purified Ssb_N or Ssb_{NC} (prey). Subsequently the columns were washed and eluted by increasing imidazole concentration (250 mM). The column elutants (lane 3: Ssb_{NC} and Ssb_C, and lane 6: Ssb_N and Ssb_C) were resolved by 14% SDS PAGE. Proteins in Lanes 2, 5 and 8 represent Ssb_{NC}, Ssb_N and Ssb_C, respectively for easy assessment of prey and bait proteins. Lane 4 contained protein molecular mass marker (SDS7, Sigma). C1 and C2 (Lane 1 and 7) represent eluted fraction of Ssb_{NC} and Ssb_N treated similarly but in absence of Ssb_C (bait) protein.

between various Deinococcal Ssb variants, we incubated the indicated concentrations of Ssb_{NC} or Ssb_N (1–5 μM) with a fixed concentration of Ssb_C (0.125 or 0.25 μM), followed by addition of oligo dT50 (0.125 μM). At a limiting concentration of only Ssb_C (0.125 μM or 0.25 μM), no measurable ssDNA binding activity was observed (lane 2 in panels A–D in Fig. 3). However, in the presence of Ssb_C (0.125 μM), the amounts of DNA–protein complexes increased with increasing concentration of Ssb_{NC} or Ssb_N (1–5 μM) (Fig. 3A and B). Also, when Ssb_C concentration was increased to 0.25 μM, the amount of DNA–protein complex increased further [Fig. 3(C, D)]. However, irrespective of the Ssb_C concentration used, interaction of Ssb_N with Ssb_C resulted in a single ssDNA–protein complex (Fig. 3A, C) while interaction of Ssb_{NC} with Ssb_C resulted in 2 distinct protein–ssDNA complexes (Fig. 3B and D). At a fixed Ssb_C concentration of 0.25 μM and 5 μM of Ssb_{NC}, we observed the formation of a well-defined Ssb_{NC}/Ssb_C complex, indicating that saturation had been achieved (Fig. 3D). Polydispersive forms of Ssb_N and Ssb_{NC} were separated by gel chromatography. A dimeric and multimeric fractions were obtained for both proteins which displayed comparable ssDNA binding capacity, either alone or in association with Ssb_C, similar to the unseparated pool of proteins (Supplementary Fig. S1). Deinococcal Ssb_C resembled *E. coli* Ssb so by using EMSA, we also tested the ability of Ssb_{NC} or Ssb_N to complement the ssDNA binding activity of *E. coli* Ssb at a limiting concentration. Ssb_{NC} or Ssb_N (3–5 μM) did not functionally complement ssDNA binding activity of *E. coli* Ssb (Fig. 3E) as no improvement in the yield of DNA–protein complexes was seen. Thus, the above data suggest that the $Ssb_{NC/N}$ specifically interacts with cognate Ssb_C only.

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

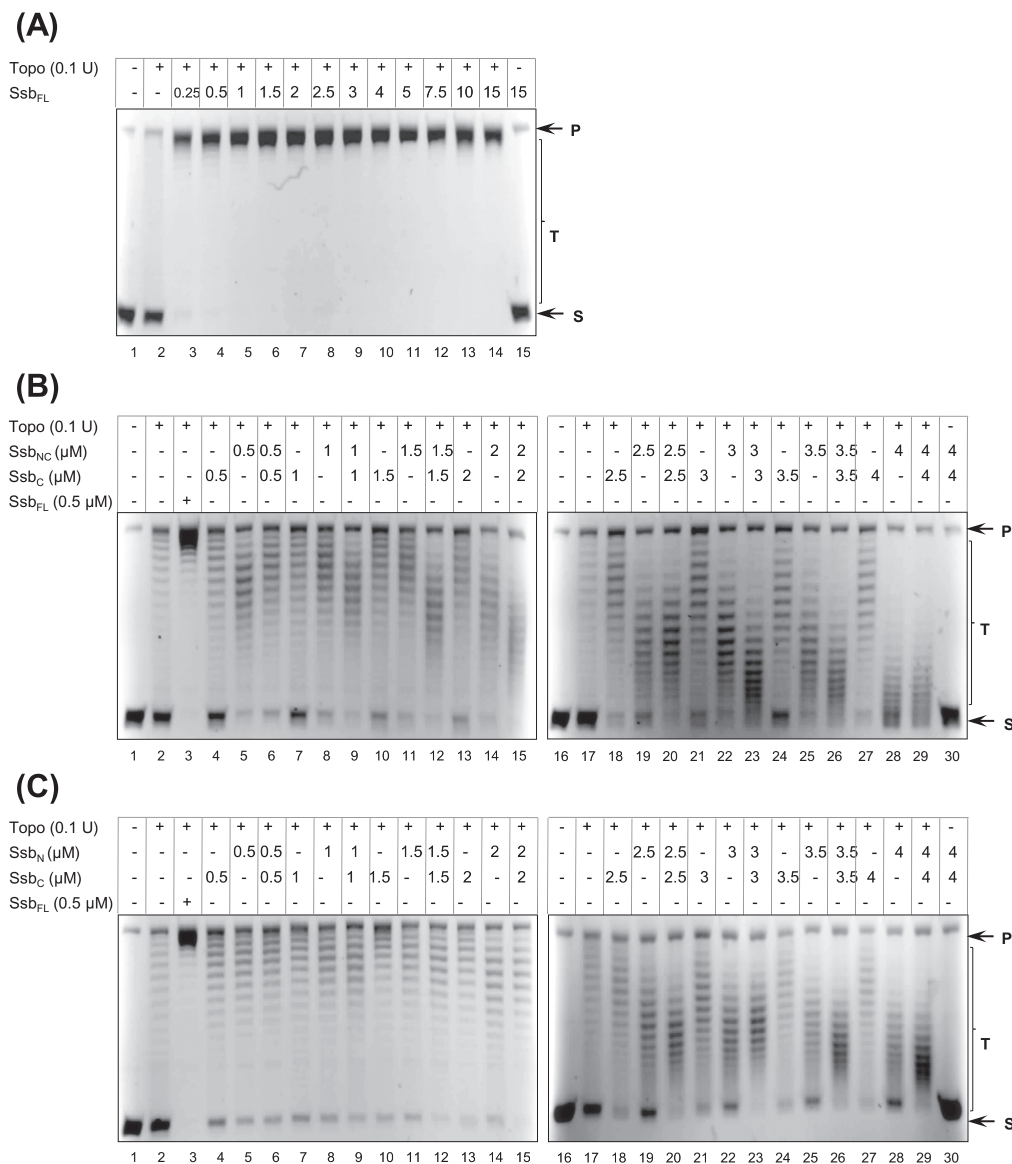

Fig. 4. Modulation of *E. coli* topoisomerase activity by Deinococcal Ssb variants: (A) supercoiled M13mp18RFI DNA was incubated with *E. coli* topoisomerase I (0.1 unit) in the presence or absence of the indicated concentration of Deinococcal Ssb_{FL} as described in Section 2. The reaction mixture was resolved on 0.8% agarose gel (23 V for 16 h) and gel was stained with ethidium bromide. The concentrations of Ssb_{FL} are indicated at the top of each lane. The 15th lane of the gel is substrate control. (B) The topoisomerase assay was performed as described above, except that Ssb_{NC} and Ssb_C were used either alone or in combination. The concentrations of various Ssb variants are indicated at the top of each lane. (C) Topoisomerase assay was performed as described above, except that Ssb_N was used instead of Ssb_{NC}. The 30th lane of both Ssb_{NC} and Ssb_N is substrate control and lane 3 contains Ssb_{FL} at 0.5 μM as positive control. Substrate, product and intermediate topoisomers are marked as "S", "P" and "T" respectively.

250 mM imidazole. Analysis of fractions indicated that Ssb_{NC} or Ssb_N co-eluted with Ssb_C (Fig. 3F, lanes 3 and 6), thereby confirming physical interaction of N-terminal domain of Deinococcal Ssb with its C-terminal domain. The observation that Ssb_{NC} or Ssb_N interact both physically and functionally with Ssb_C suggest that correct folding of the whole complex leads to higher affinity for ssDNA as compared to Ssb_C alone (Figs. 2 and 3). Both the complexes showed different binding which could be attributable to the connector region via additional protein–protein interactions.

3.5. Ssb variants enhance topoisomerase I activity but do not stimulate DNA strand exchange promoted by Deinococcal RecA

Ssb is known to enhance the catalytic activity of topoisomerase I by localized melting of ssDNA. This property is utilized to test limited DNA melting activity of DNA binding proteins, including Ssb, in a standard topoisomerase assay that employs heterologous topoisomerase [30,39–41]. We tested the effect of Deinococcal Ssb and its variant forms on *E. coli* topoisomerase I activity using dsM13DNA. Topoisomerase I formed a single major band of relaxed DNA in presence of Ssb_{FL} [Fig. 4A (lanes 3–14), Fig. 4B and C – lane 3], but in the presence of Ssb_C, Ssb_{NC} or Ssb_N we observed the generation of DNA topoisomers. In the presence of N-terminal domains (Ssb_{NC} and Ssb_N) topoisomerase I generated intermediate topoisomers at lower concentration of Ssb_{NC} and Ssb_N (Fig. 4B and C, lane 5, 8, 11, 14), while exerted inhibition at higher concentrations of these proteins. In the presence of Ssb_C, topoisomerase I resulted in a higher yield of relaxed DNA at higher concentration than at lower concentration of Ssb_C (Fig. 4B and C, lane 18, 21, 24, 27). The data indicate that both the OB folds of Deinococcal Ssb were independently capable of melting ssDNA locally, thereby enhancing topoisomerase I activity. However, Ssb_{NC}/Ssb_N appeared to be self-inhibiting at higher concentrations and their addition decreased the positive effect of Ssb_C on

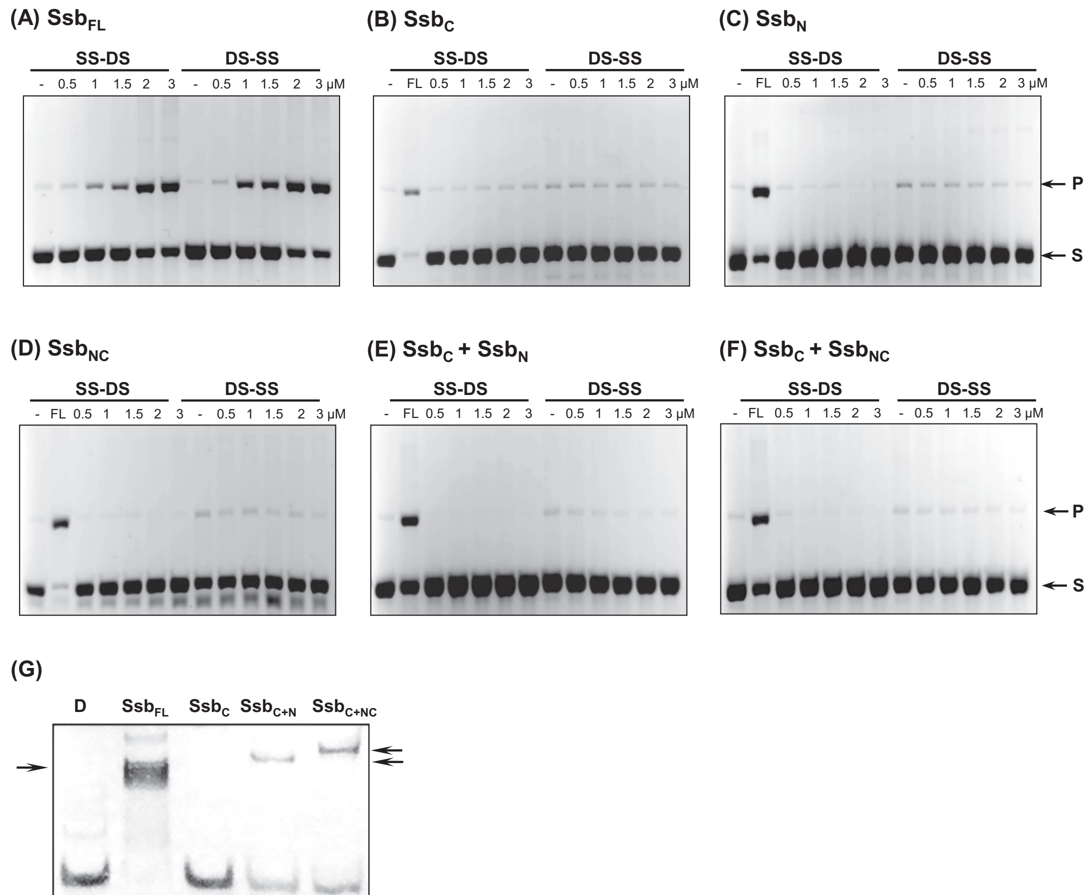

Fig. 5. DNA strand exchange assay. Assay was performed with Deinococcal RecA (3 μM) in the presence of (A) Ssb$_{FL}$, (B) Ssb$_{C}$ (C) Ssb$_{N}$ (D) Ssb$_{NC}$ (E) Ssb$_{N}$ + Ssb$_{C}$ and (F) Ssb$_{NC}$ + Ssb$_{C}$ (at concentrations indicated on top of each panel) in SS–DS or DS–SS mode. Lane "-", in all the gels contains RecA but do not contain any Ssb variants. Lane FL in figure (B)–(F) contains Ssb$_{FL}$ (2 μM) as a positive control. Substrate and product are marked as "S" and "P" respectively. (G) EMSA carried out in strand exchange assay conditions. Lane 1 contains only ssDNA, lane 2 contains Ssb$_{FL}$ at 0.5 μM, lane 3 contains Ssb$_{C}$ at 0.5 μM, lane 4 contains Ssb$_{C}$ and Ssb$_{N}$ at 0.5 μM each and lane 5 contains Ssb$_{C}$ and Ssb$_{NC}$ at 0.5 μM each.

topoisomerase I activity. The modulation seen in topoisomerase activity may be due to stabilization of single-stranded DNA formed at the site of topoisomerase action. The variation seen in the pattern of DNA topoisomers generated by Ssb$_{NC/N}$ or Ssb$_{C}$ may be attributable to the affinity for single-stranded portion formed at topoisomerase action site as well as accessibility to such sites. Following gamma irradiation, accumulation of high concentration Ssb protein and its C-terminal processing is well documented [9,21]. The data reported here suggest that even the Ssb variants would be able to enhance the activity of native topoisomerases during DNA damage repair in the recovery phase.

We next tested the ability of Deinococcal Ssb variants to stimulate DNA strand exchange promoted by the cognate RecA. RecA carries out strand exchange from linear double stranded DNA onto circular ssDNA (substrates) leading to generation of nicked circular (product) molecule. In SS–DS mode, RecA was first incubated with ssDNA followed by addition of Ssb and dsDNA was added last. In DS–SS mode, RecA was first incubated with dsDNA followed by addition of ssDNA and Ssb was added at the end. Ssb plays a very important role in promoting strand exchange assay by removing secondary structure of circular ssDNA. Addition of Ssb$_{FL}$ up to 3 μM increased the efficiency of strand exchange as seen by the extent of the formation of nicked circular DNA, both in the SS–DS mode as well as in the DS–SS mode (Fig. 5A). However, addition of Ssb$_{C}$, Ssb$_{N}$ or Ssb$_{NC}$ individually failed to enhance product formation, in either SS–DS or DS–SS modes of strand exchange (Fig. 5B–D). *In vitro* combination of Ssb$_{N}$ or Ssb$_{NC}$ with Ssb$_{C}$ also did not augment product formation (Fig. 5E, F). Competitive strand exchange assays carried out in combination of Ssb$_{FL}$ with all other variants (Ssb$_{C}$, Ssb$_{NC}$ and Ssb$_{N}$) did not inhibit the progress of strand exchange reaction (data not shown). EMSA carried out under strand exchange conditions showed the formation of prominent DNA–protein complexes of Ssb$_{NC}$ or Ssb$_{N}$ and Ssb$_{C}$ (Fig. 5G). Thus, the N-terminal and C-terminal Ssb variants did form expected DNA–protein complexes under strand exchange assay condition but were unable to remove secondary structures of virion ØX174 ssDNA. Ssb$_{FL}$ binds to ssDNA with highest affinity compared to all the variants of Ssb and is capable of removing such secondary structures as it contains naturally linked N-terminal and C-terminal portions by connector region thus enhancing RecA activity (Fig. 5).

4. Conclusion

Deinococcus-Thermus group of bacteria, known to thrive in extreme environments that readily cause breach in DNA integrity, encode a novel Ssb composed of 2 asymmetric OB folds linked by a 10 amino acid long connector. The evolution of nearly twice the size of Ssb protein in these bacteria, in comparison to other prokaryotes, suggests a specialized function. Bioinformatic analyses together with crystal structure data have earlier implicated C-terminal OB fold in ssDNA binding [26] with a few residues of N-terminal OB fold also taking part in ssDNA binding [9]. The data presented here clearly demonstrate the functional significance of

individual OB folds in the novel Deinococcal Ssb protein. The ssDNA binding capability primarily resides in C-terminal OB fold while the N-terminal OB fold is engaged in multimerisation and, together with C-terminal OB fold, displays improved ssDNA binding *in vitro*. However, specific interactions and cooperative contribution of both the OB folds in Ssb_{FL} is necessary for efficient melting of secondary structures in ssDNA, a feature essential for DNA replication, recombination and repair. Evolution of such atypical Ssb appears to be prompted by a necessity to protect as much ssDNA templates as possible, following stress-induced massive DNA damage. Cooperative interactions between the multimerization inducing N-terminal OB fold and the ssDNA binding C-terminal OB fold appear to functionally complement each other to accomplish rapid nucleation and thereby, protection of ssDNA templates to be used for efficient and error-free DNA repair in Deinococci.

Author contribution statement

AU planned and performed experiment and wrote paper. BB planned the experiment and analyzed data. KM and SKA visualized the theme and approach, wrote and corrected the paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fob.2015.04.009.

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