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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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 DdR, which is the other ssDNA binding protein found in Deinococcal species [23].Biochemical analyses have revealed
that Deinococcal Ssb displays salt-independent weak ssDNA binding activity and displaces shorter strand of duplex DNA than E. coli Ssb [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$_4$ 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 (SsbFL, amino acids 1–301), N-terminal OB domain (SsbNC, amino acids 1–114), N-terminal OB domain with connector (SsbNC, amino acids 1–124) and C-terminal OB domain (SsbC, 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, phosphocholine 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$_{ds}$, ssb$_{nc}$ and ssb$_{C}$) 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$_{ds}$) was amplified using specific primers FLF and FLR (Table 1) digested with BamHI and ligated to pUC19 at BamHI site to obtain plasmid pUC19SsbFL. The insert from pUC19SsbFL was released by digesting it with Ndel and BamHI and then ligating it to pET16b at corresponding sites to generate plasmid pETSsbFL. Ssb-C-terminal domain (SsbC) was PCR amplified by using primers CTF and CTR and cloned at Ndel and BamHI restriction sites of pTE16b to generate plasmid pETSsbC. Ssb-N-terminal (SsbNC) domain with connector was amplified by using primers NCF and NCD and cloned at Ndel and BamHI restriction sites of pTE16b to generate plasmid pETSsbNC. RecA gene was amplified from E. coli K12 genomic DNA as previously described [18]. The insert from pET16b was released by digesting it with Ndel and BamHI and then ligating it to pET16b to generate plasmid pET16b-RecA. RecA gene was cloned at Ndel and BamHI restriction sites of pTE16b to generate plasmid pETSsbC-RecA.

2.3. Recombinant protein expression

Select Bacterial strains and plasmids 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 DH5x                  | F’ endA1 glv44 thy-1 recA1 gyrA96 deoR60 supE44 U169 A(lacZYA-argF)3169, hsdR17(rK mQ)12, LAM15 | Lab collection |
| E. coli BL-21(pLysS)          | F’ ompT glnX53 deoR60 supE44 U169 A(lacZYA-argF)3169, hsdR17(rK mQ)12, LAM15 | Novagen |
| E. coli ER2566                | fhuA2 lacZ77 gene1 [lon] ompT glnX53 deoR60 supE44 U169 A(lacZYA-argF)3169, hsdR17(rK mQ)12, LAM15 | NEB |
| E. coli STL2669               | (recA-sir)306, Tn50 xonA2 (sbeB) | Lab collection |
| E. coli BLSsb$_{ds}$          | E. coli BL-21(pLysS) cells containing recombinant plasmid pET16b-Ssb$_{ds}$ | This study |
| E. coli BLSsb$_{C}$           | E. coli BL-21(pLysS) cells containing recombinant plasmid pET16b-Ssb$_{C}$ | This study |
| E. coli ERSsb$_{NC}$          | E. coli ER2566 cells containing recombinant plasmid pET16b-Ssb$_{NC}$ | This study |
| E. coli ERSsb$_{NC}$          | E. coli ER2566 cells containing recombinant plasmid pET16b-Ssb$_{NC}$ | 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$_{ds}$                | Deinococcal Ssb$_{ds}$ gene cloned at NdeI/BamHI restriction sites | This study |
| pETSsb$_{C}$                 | C-terminal region of Deinococcal Ssb cloned at NdeI/BamHI | This study |
| pETRecA                      | Deinococcal RecA gene cloned at Ncol/BamHI restriction sites | This study |
| pTWIN5Nsb$_{NC}$             | N-terminal with connector region of Deinococcal Ssb cloned at EcoRI/BamHI restriction sites | This study |
| pMXB10Ssb$_{ds}$             | N-terminal region of Deinococcal Ssb cloned at Ndel/Xhol restriction sites | This study |
| **Primers**                  |             |                             |
| FLF                          | 5’-CCAGTGATCCAGGAGGAAATTTTGGCATATTCG-3 | BamHI, NdeI |
| FLR                          | 5’-CCAGTGATCCAGGAGGAAATTTTGGCATATTCG-3 | BamHI |
| CTF                          | 5’-TCAAGTCGATCATGCGCCGCGCGGATATAGC-3 | NdeI |
| CTR                          | 5’-TCAAGTCGATCATGCGCCGCGCGGATATAGC-3 | BamHI |
| NCF                          | 5’-CCAGTGATCCAGGAGGAAATTTTGGCATATTCG-3 | EcoRI |
| NCD                          | 5’-CCAGTGATCCAGGAGGAAATTTTGGCATATTCG-3 | BamHI |
| NF                           | 5’-CCAGTGATCCAGGAGGAAATTTTGGCATATTCG-3 | NdeI |
| ND                           | 5’-CCAGTGATCCAGGAGGAAATTTTGGCATATTCG-3 | Xhol |
| RF                           | 5’-CCAGTGATCCAGGAGGAAATTTTGGCATATTCG-3 | NdeI |
| RD                           | 5’-CCAGTGATCCAGGAGGAAATTTTGGCATATTCG-3 | Xhol |

Restriction site contained in primer is underlined.
2.3. Over expression and purification of SsbFL and SsbC

The constructs pETSsbFL and pETSsbC were transformed separately into E. coli BL21 (pLysS) cells to obtain strains BLSsbFL and BLSsbC. 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–nitrilotriacetic 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 dialedyzed in Ssb storage buffer, snap-frozen in liquid nitrogen and stored at −80 °C.

2.4. Over expression and purification of SsbNC and SsbN

The pTwinSsbNC and pMXB10SsbN constructs were separately transformed into E. coli strain ER2566 to obtain ERSsbNC and ERSsbN. 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 8.3, 0.5 M NaCl) were loaded onto a chitin column and washed with 20 volumes of column buffer (20 mM Tris, pH 8.3, 0.5 M NaCl). On-column cleavage of SsbNC 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 SsbN 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 SsbNC or SsbN were passed through Q-Sepharose column and eluted by a (20 mM Tris–acetate pH 7.4, 1 mM EDTA, 1 mM 2-mercaptoethanol and 50% glycerol), 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 40,000g for 1 h. RecA protein was precipitated by adding 10% Polymin-P (pH 7.9) to a final concentration of 0.5% over 15 min with continuous stirring. The pellet was extracted until 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. SsbFL or SsbC eluted as single major peak (fraction F1). SsbN or SsbNC 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 SsbFL, SsbN, SsbNC or SsbN 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 MnCl2 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-bromo mo-4-chloro-3-indophenyl) colorimetric substrate. The bands formed were quantitated by gel quant software (Biosystematica, UK) and data was fitted to Hill’s equation to obtain K0 value for SsbN and SsbC. Each experiment was repeated three times and K0 values were obtained from each experiment. The K0 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 (SsbNC or SsbN and SsbC) and also with combination of SsbNC/SsbN and E. coli Ssb.

2.8. Domain interaction studies

Purified SsbFL was bound to Ni–NTA matrix through its His tag. SsbNC or SsbN was then added to Ni–NTA attached SsbL separately. As a control, SsbNC or SsbN 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 eluant so obtained were resolved by 14% SDS–PAGE and stained with Coomassie blue.

2.9. Stimulation of E. coli topoiso- merase 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 ssDNA.
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 lM 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 SsbNC shares 33% identity and 51% similarity with SsbC and 38% identity and 49% similarity with E. coli Ssb (Blastp analysis, data not shown). SsbC shared 39% identity and 64% similarity with E. coli Ssb protein [16] and is similar to E. coli Ssb than to SsbNC. 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. SsbFL and SsbC were expressed with Histidine Tag. SsbNC and SsbN 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 variant forms and Deinococcal RecA were cloned, over-expressed in E. coli and purified as detailed under Section 2. SsbFL and SsbC were expressed with Histidine Tag. SsbNC and SsbN 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.

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 SsbFL, SsbC, SsbNC, SsbN 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 (A, Bovine Serum Albumin: 66 kDa; B, Ovalbumin: 44 kDa; C, Carbonic anhydrase: 29 kDa; D, 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 SsbNC and SsbN could not be determined. (D) Fractions of Ssb variants resolved by 14% SDS PAGE. The fraction number of all Ssb variants is shown.
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 SsbNC 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 SsbN dimeric profile could be contributed by small fraction of monomeric protein of SsbN, 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 SsbFL.

3.3. Ssb domains exhibit differential ssDNA binding

The ssDNA binding activity of Deinococcal Ssb protein variants was tested by Electrophoretic Mobility Shift Assay (EMSA) using 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 SsbN and SsbC using Gel Quant software. The representative graph for SsbN [panel A (ii)] and SsbC [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) SsbFL (B) SsbC (C) SsbN (D) SsbNC.

![Graphs showing ssDNA binding activity of SsbFL, SsbC, SsbN, and SsbNC](image)

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 SsbN and SsbC using Gel Quant software. The representative graph for SsbN [panel A (ii)] and SsbC [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) SsbFL (B) SsbC (C) SsbN (D) SsbNC.

3.4. SsbN/SsbNC and SsbC display physical and functional interaction in vitro

The functional and physical interactions were determined by EMSA and affinity chromatography. To test direct interaction
between various Deinococcal Ssb variants, we incubated the indicated concentrations of SsbNC or SsbN (1–5 µM) with a fixed concentration of SsbC (0.125 or 0.25 µM), followed by addition of oligo dT50 (0.125 µM). At a limiting concentration of only SsbC (0.125 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 SsbC (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 SsbC concentration was increased to 0.25 µM, the amount of DNA–protein complex increased further [Fig. 3(C, D)]. However, irrespective of the SsbC concentration used, interaction of SsbN with SsbC resulted in a single ssDNA–protein complex (Fig. 3A, C) while interaction of SsbNC with SsbC resulted in 2 distinct protein–ssDNA complexes (Fig. 3B and D). At a fixed SsbC concentration of 0.25 µM and 5 µM of SsbNC, we observed the formation of a well-defined SsbNC/SsbC complex, indicating that saturation had been achieved (Fig. 3D). Polydispersive forms of SsbN and SsbNC 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 SsbC, similar to the unseparated pool of proteins (Supplementary Fig. S1).

Deinococcal SsbC resembled E. coli Ssb so by using EMSA, we also tested the ability of SsbNC or SsbN to complement the ssDNA binding activity of E. coli Ssb at a limiting concentration. SsbNC or SsbN (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 SsbNC/N specifically interacts with cognate SsbC only.

Direct interaction of SsbNC or SsbN with SsbC was ascertained, in vitro, by affinity chromatography. SsbC was first bound to Ni–NTA by its His-tag and purified SsbNC or SsbN (without any tag) were then individually passed through the column. After extensive washing, the column was eluted by increasing imidazole concentration (250 mM). The column elutants (lane 3: SsbNC and SsbC) were resolved by 14% SDS PAGE. Proteins in Lanes 2, 5 and 8 represent SsbNC, SsbN and SsbC, 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 SsbNC and SsbC treated similarly but in absence of SsbC (bait) protein.

Fig. 3. In vitro functional interactions of Deinococcal SsbN/NC with SsbC. (A,B) EMSA was carried out as detailed in Fig. 2 except that indicated concentration of SsbN (A) and SsbNC (B) were mixed with fixed concentration of SsbC (0.125 µM) and then incubated with 0.125 µM oligo dT50. The presence or absence of SsbC protein is indicated by “+” or “−” sign above each lane. (C,D) EMSA was carried out as detailed in (A, B) except that concentration of SsbNC was fixed at 0.25 µM. (E) EMSA was carried out as detailed in (A) except that SsbC 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. SsbC (bait) was bound to Ni–NTA by its His-tag and the column was equilibrated with purified SsbN or SsbNC (prey). Subsequently the columns were washed and eluted by increasing imidazole concentration (250 mM). The column elutants (lane 3: SsbNC and SsbC, and lane 6: SsbN and SsbC) were resolved by 14% SDS PAGE. Proteins in Lanes 2, 5 and 8 represent SsbNC, SsbN and SsbC, 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 SsbNC and SsbC treated similarly but in absence of SsbC (bait) protein.
250 mM imidazole. Analysis of fractions indicated that SsbNC or SsbN co-eluted with SsbC (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 SsbNC or SsbN interact both physically and functionally with SsbC suggests that correct folding of the whole complex leads to higher affinity for ssDNA as compared to SsbC 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 SsbFL [Fig. 4A (lanes 3–14), Fig. 4B and C – lane 3], but in the presence of SsbC, SsbNC or SsbN we observed the generation of DNA topoisomers. In the presence of N-terminal domains (SsbNC and SsbN) 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 SsbC, topoisomerase I resulted in a higher yield of relaxed DNA at higher concentration than at lower concentration of SsbC (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, SsbNC/SsbN appeared to be self-inhibiting at higher concentrations and their addition decreased the positive effect of SsbC on
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 SsbNC/N or SsbC 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 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 SsbC, SsbN or SsbNC individually failed to enhance product formation, in either SS–DS or DS–SS modes of strand exchange (Fig. 5B–D). In vitro combination of SsbN or SsbNC with SsbC also did not augment product formation (Fig. 5E, F). Competitive strand exchange assays carried out in combination of SsbFL with all other variants (SsbC, SsbNC and SsbN) 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 SsbNC or SsbN 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

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 Deinococcus 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 ssDNA binding in vitro. However, specific interactions and cooperative contribution of both the OB folds in Ssb$_B$ 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 multimerisation 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.febb.2015.04.009.

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