

Identification and properties of the *Deinococcus grandis* and *Deinococcus proteolyticus* single-stranded DNA binding proteins (SSB)

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To study the biochemical properties of SSB's from *Deinococcus grandis* (*DgrSSB*) and *Deinococcus proteolyticus* (*DprSSB*), we have cloned the *ssb* genes obtained by PCR and have developed *Escherichia coli* overexpression systems. The genes consist of an open reading frame of 891 (*DgrSSB*) and 876 (*DprSSB*) nucleotides encoding proteins of 296 and 291 amino acids with a calculated molecular mass of 32.29 and 31.33 kDa, respectively. The amino-acid sequence of *DgrSSB* exhibits 45%, 44% and 82% identity and the amino-acid sequence of *DprSSB* reveals 43%, 43% and 69% identity with *Thermus aquaticus* (*TaqSSB*), *Thermus thermophilus* (*TthSSB*) and *Deinococcus radiodurans* SSBs, respectively. We show that *DgrSSB* and *DprSSB* are similar to *Thermus/Deinococcus* SSBs in their biochemical properties. They are functional as homodimers, with each monomer encoding two single-stranded DNA binding domains (OB-folds). In fluorescence titrations with poly(dT), both proteins bind single-stranded DNA with a binding site size of about 33 nt per homodimer. In a complementation assay in *E. coli*, *DgrSSB* and *DprSSB* took over the *in vivo* function of *EcoSSB*. Thermostability with half-lives of about 1 min at 65–67.5°C make *DgrSSB* and *DprSSB* similar to the known SSB of *Deinococcus radiodurans* (*DraSSB*).

Keywords: SSB, DNA replication, expression, purification, bacteria

INTRODUCTION

Single-stranded DNA-binding (SSB) proteins play central roles in cellular and viral processes involving the generation of single-stranded DNA. These include DNA replication, DNA repair pathways and homologous recombination (Shamoo *et al.*, 1995; Webster *et al.*, 1997; Wadsworth & White, 2001; Kur *et al.*, 2005). SSBs bind DNA using four 'OB-fold' (oligonucleotide/oligosaccharide-binding fold) domains (Murzin, 1993) that can be organized in a variety of overall quaternary structures. They are found in different organisms as homotetramers (most prokaryotic, mitochondrial and crenarchaeal SSBs), homodimers (SSBs from bacteriophages, *Deinococcus-Thermus* phylum of bacteria), heterotrimers (euryarchaeal and eucaryal RPAs)

(Williams & Konigsberg, 1978; Shamoo *et al.*, 1995; Stassen *et al.*, 1995; Webster *et al.*, 1997; Wadsworth & White, 2001; Dąbrowski *et al.*, 2002a; Bernstein *et al.*, 2004; Kur *et al.*, 2005). Although there is little conservation at sequence level, the crystal structures of human RPA (Bochkarev *et al.*, 1997; 1999), *Escherichia coli* (Webster *et al.*, 1997; Raghunathan *et al.*, 2000), *Deinococcus radiodurans* (Bernstein *et al.*, 2004), *Thermus aquaticus* (Jedrzejczak *et al.*, 2006) and human mitochondrial (Yang *et al.*, 1997) SSBs indicate the presence of a core 'OB-fold'. This domain comprises approximately 100 amino acids and is responsible for the major interactions with single-stranded DNA. The universality of OB-fold domains in SSB proteins suggests that all extant SSB and RPA proteins are derived from a common ancestral SSB.

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Nucleotide sequence accession number. The nucleotide sequences of the *ssb* genes are available in the Gen Bank database under accession numbers: for *D. grandis*: DQ857725 and for *D. proteolyticus*: DQ979351.

Abbreviations: dsDNA, double-stranded DNA; IPTG, isopropyl β -D-thiogalactoside; OB fold, oligonucleotide/oligosaccharide-binding fold; RPA, replication protein A; SSB, single-stranded DNA binding protein.

Although, virtually all bacterial SSB family members act as homotetramers, recent discoveries have shown that SSB proteins from *Deinococcus-Thermus* phylum of bacteria adopt a different architecture. They are functional as homodimers, with each SSB monomer encoding two OB folds linked by a conserved spacer sequence (Dąbrowski *et al.*, 2002a; Bernstein *et al.*, 2004; Kur *et al.*, 2005). Homodimeric SSBs, contrary to tetrameric SSB proteins, possess only two C-terminal tails in each active form. Reducing the number of C-terminal tails by half can dramatically affect the function of SSB protein containing two OB-folds *in vivo* (Curth *et al.*, 1996). Probably the fusion of two OB folds linked by a conserved spacer sequence is an adaptation of *Deinococcus-Thermus* to extreme conditions (Dąbrowski *et al.*, 2002a; 2002b; Bernstein *et al.*, 2004). Bacteria from this group thrive in extreme environments that would kill most cells (desiccation, severe DNA-damaging and/or high-temperature conditions) by using mechanisms that are presently unclear.

At the present time there are twenty validly described *Deinococcus* species (Hirsch *et al.*, 2004; de Groot *et al.*, 2005; Omelchenko *et al.*, 2005). With the exception of *D. murrayi* and *D. geothermalis*, which have optimum growth temperatures of 45 to 50°C, all other species have optimum growth temperatures between about 25 to 35°C (Ferreira *et al.*, 1997). The most striking characteristic of the species of this genus is their extreme resistance to UV and gamma radiation. Two *Deinococcus* genome sequences are currently available (*D. radiodurans* and *D. geothermalis*) and SSBs of *D. radiodurans*, *D. radiopugnans* and *D. geothermalis* have been described in detail (Bernstein *et al.*, 2004; Witte *et al.*, 2005).

At present three *Deinococcus* SSB-like proteins have been described in detail. They are SSBs from *D. radiodurans* (Bernstein *et al.*, 2004; Witte *et al.*, 2005), *D. geothermalis* (Filipkowski *et al.*, 2006a) and *D. radiopugnans* (Filipkowski *et al.*, 2006b). Here we report the identification, purification and characterization of new SSB proteins from other *Deinococcus* species (*D. grandis* and *D. proteolyticus*), and their relationship with other members of this important class of proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, enzymes, and reagents. *D. grandis* DSMZ 3963 and *D. proteolyticus* DSMZ 20540 strains were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany). *E. coli* TOP10F' strain and the pBADMyHisA plasmid were from Invitrogen (USA) and were used for protein expression. The reagents for PCR and various oligodeoxy-

nucleotides were obtained from DNA-Gdańsk II (Poland). DNA from *D. grandis* and *D. proteolyticus* were isolated using Genomic DNA Prep Kit (A&A Biotechnology, Poland).

Identification of *D. grandis* and *D. proteolyticus* *ssb* genes. Based on the known localization of the *ssb* gene in *D. radiodurans* (GenBank Accession No. AY293617) and *D. geothermalis* 11300 (GenBank Accession No. NC008025 and CP000359, previously: AAHE01000002) genomes, where the *ssb* gene is flanked by the conservative *rpsF* and *rpsR* genes encoding the ribosomal proteins S6 and S18, primers complementary to those genes for PCR amplification were designed and synthesized. The forward primer used for amplification of DNA containing the *ssb* gene of *D. grandis* was: 5'-GACAACGTC-CGCCGCGTCCTGGTGGT (26 nt) and the reverse primer was: 5'-GTGCGGCGGGCGGGAAGAATCTT (23 nt) (Filipkowski *et al.*, 2006a). The forward primer used for PCR amplification of DNA containing the *ssb* gene of *D. proteolyticus* was: 5'-GCCCR-GARTGGAAGACCAAGAAGGC (25 nt) and the reverse primer was: 5'-GCGGCGGGGAAGAATCTT-GCC (21 nt) (Filipkowski *et al.*, 2006b). PCRs were carried out using proofreading DNA polymerase *Pwo* (DNA-Gdańsk II, Poland) and 35 cycles with a temperature profile of 60 s at 95°C, 60 s at 68°C, and 90 s at 72°C. In both cases specifically, about 1200–1300 bp PCR products were obtained and then were sequenced to confirm the presence of *ssb*-like gene.

The obtained *ssb* gene sequences of *D. grandis* and *D. proteolyticus* were used to design gene-specific primers for PCR and cloning into pBADMyHisA vector. PCR was carried out using forward 5'-ATAC-CATGGCCCGAGGCATGAACCA-3' and reverse 5'-AAAGCTTCAGAACGGCAGGTCCTCGTC-3' primers for *ssb* of *D. grandis* and forward 5'-TATAC-CATGGCCCGAGGAATGAATCACGTT-3' and reverse 5'-ACTAAGCTTCAAACGGCATATCGTCT-TCTTCTGG-3' primers for *ssb* of *D. proteolyticus*. The boldface parts of primer sequences are complementary to the nucleotide sequences of the *ssb* gene, whereas 5' overhanging ends of primers contain recognition sites for restriction endonucleases and are designed to facilitate cloning (*Nco*I or *Hind*III sites, underlined; start and stop codons, italicized). For *D. grandis* *ssb* amplification 25 cycles were performed with a temperature profile of 60 s at 94°C, 60 s at 68.4°C, and 120 s at 72°C and for *D. proteolyticus* *ssb* amplification 30 cycles were performed with a temperature profile of 60 s at 94°C, 60 s at 67.7°C, and 120 s at 72°C.

Cloning of *D. grandis* and *D. proteolyticus* *ssb* genes. The obtained PCR products were digested with *Nco*I and *Hind*III, and fractionated on 1.5% agarose gel. DNA corresponding to 902 bp for *D. grandis* *ssb* and 890 bp for *D. proteolyticus* *ssb* were



cut out of the gel and purified using Gel-Out Kit (A&A Biotechnology, Poland). In both cases the purified fragments were ligated into pBADMyHisA between *Nco*I and *Hind*III sites. *E. coli* TOP10F' cells were transformed with the ligation mixture and obtained colonies were examined for the presence of *ssb* gene of *D. grandis* or *D. proteolyticus* by PCR amplification and restriction analysis. The resulting plasmid was referred to as pBADD_{gr}SSB or pBAD-D_{pr}SSB. The authenticity of the clones was ascertained by sequencing.

Expression and purification. SSBs were over-expressed using pBADD_{gr}SSB or pBADD_{pr}SSB in *E. coli* TOP10F' as described previously (Filipkowski *et al.*, 2006a) with some modifications. Cells were grown in 500 ml culture at 37°C to an OD₆₀₀ of 0.6 in LB-broth containing 100 µg/ml ampicillin. The expression of SSBs was induced with arabinose at a final concentration of 0.2% for D_{gr}SSB and 0.02% for D_{pr}SSB. Cells were harvested after additional 4 h culturing by centrifugation and suspended in 30 ml buffer A consisting of 10 mM Tris/HCl, pH 9, 10 mM EDTA, 10 mM CaCl₂, 0.5 mg/ml lysozyme, and lysed by sonication.

The purification procedure was analogous to the previously published purification scheme for thermostable SSB-like proteins (Dąbrowski *et al.*, 2002) with some modifications in the first step of procedure (heat treatment). The reduction of contamination by the host proteins after heat treatment was carried out at 57.5°C for 10 min. The purity of proteins was examined by SDS/PAGE. The amounts of the purified SSB protein were estimated spectrophotometrically using the appropriate absorption coefficient factor and by the optical densitometry on SDS/PAGE gel with Quantity One program (VersaDoc, BioRad).

The cloning, expression and purification procedure for SSB from *D. radiodurans* (D_{ra}SSB) was analogous to the previously published scheme by Eggington *et al.* (2004).

Analytical gel filtration chromatography. D_{gr}SSB and D_{pr}SSB proteins (up to about 1 mg total) were subjected to gel filtration chromatography on a Superdex HR 75 column (Amersham Bioscience) equilibrated with 60 ml buffer containing 20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 10 mM EDTA, and were eluted with the same buffer at a flow rate of 0.5 ml/min and were collected in 2.5 ml fractions. Absorbance at 280 nm was recorded to monitor the elution profile. The elution patterns of SSB proteins were then compared with those of standard proteins: bovine albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and cytochrome *c* (12.4 kDa).

Agarose gel electrophoretic retardation analysis. The fluorescein-labelled ss dT-oligonucleotides

35, 60, 70 or 76 nucleotides in length (10 pM) were incubated with a known concentration of purified SSB protein in binding buffer (20 mM Tris/HCl, pH 7.5, 10 mM EDTA) containing 2 or 100 mM NaCl. After 20 min at 25°C samples were loaded onto 2% agarose gels and separated by electrophoresis in 1×TAE buffer. The complexes and the free oligonucleotides were visualized under UV light.

Fluorescence titrations. The fluorimetric titration experiments were performed on a Perkin-Elmer LS-5B luminescence spectrometer as described earlier (Dąbrowski & Kur, 1999). The tryptophan emission spectra of the examined SSB proteins were obtained by excitation at 295 nm in reaction buffer (20 mM Tris/HCl, pH 7.5, 10 mM EDTA) containing 2 or 100 mM NaCl at 25°C. Fluorescence quenching at the emission peak of 348 nm was monitored whilst titrating (dT)₆₀ oligonucleotide into a fixed concentration of SSB protein. Relative fluorescence values were corrected for dilution effects and subtraction of fluorescence due to buffer and DNA components.

Thermostability. A fixed quantity (10 pM) of 5'-end fluorescein-labelled oligonucleotide (dT)₃₅ was added to 10 pM of D_{gr}SSB, D_{pr}SSB, D_{ra}SSB or T_{aq}SSB samples preincubated for 0, 1, 5, 8, 10, 30, 20, 30, 40, 60, and 90 min at 50, 55, 60, 65 and 67.5°C in 10 µl reaction mixtures in standard buffer B. After 20 min incubation at 25°C protein-DNA complexes were separated from free DNA by 2% agarose gel electrophoresis, and 50% quantities of protein-(dT)₃₅ complex were evaluated by densitometric analysis using VersaDoc imaging system and QuantityOne software (BioRad).

Complementation analysis. *E. coli* RDP268 strain, in which the chromosomal *ssb* gene is replaced by a kanamycin resistance (Δ *ssb*::Kan^R) and harboring pRPZ146 (ColEI *ori*, Tc^R) plasmid coding the wild-type SSB protein (Porter & Black, 1991) was lysogenized with λ DE3 using λ DE3 Lysogenization Kit (Novagen). The resulting strain can be used to express target genes cloned in the pET vector under the control of the T7 promoter. pRPZ146 is essential for the survival of the cells and can be replaced by another plasmid, only if it contains a gene whose product can take over *Eco*SSB function *in vivo*. We used the modified *E. coli* RDP268(DE3) strain to transform the plasmids pET23D(+)*Dgr*SSB, pET23D(+)*Dpr*SSB or pET23D(+)*Eco*SSB, which carries the *ssb* genes under the control of the T7 promoter and confers ampicillin resistance. Strains were grown in the presence of ampicillin (100 µg/ml) and kanamycin (25 µg/ml) — present during all steps. After four consecutive overnight subculturings in 3 ml of 2YT (1.6% tryptone, 1% yeast extract and 0.5% NaCl with or without 1mM IPTG) containing the same antibiotics, the colonies were plated on 2YT



agar plates containing ampicillin alone (100 µg/ml) and on plates containing both tetracycline (25 µg/ml) and ampicillin (100 µg/ml). At the same time, from the last liquid steps, we isolated (parallel to inoculating the plates) plasmids to control form (size) actually presented inside the cells. An Ap^R and Tc^S phenotype shows that the incoming plasmid harbors a gene that complements *EcoSSB*. On the other hand, maintenance of Tc^R, Ap^R phenotype indicates that the tester plasmid is unable to complement *EcoSSB*.

Protein sequence analysis. The amino-acid sequences of *DgrSSB* and *DprSSB* were analysed using standard protein-protein BLAST and RPS-BLAST. Multiple sequence alignment was generated by using the program ClustalX. The results were prepared using the editor program Gendoc (copyright Karl Nicholas).

RESULTS

Comparison of *Thermus/Deinococcus* SSBs

The nucleotide sequence of the PCR products obtained by amplification of the region between *rpsF* and *rpsR* genes revealed a contiguous ORF that encodes a complete SSB protein of *D. grandis* or *D. proteolyticus*. The monomer of the *DgrSSB* protein contains 296 amino-acid residues and *DprSSB* 291 amino-acid residues and they are five and ten amino-acid residues respectively, shorter than the largest bacterial SSB polypeptide identified up to date (*D. radiodurans* SSB contains 301 amino-acid residues). Analysis of the primary structures revealed the presence of two putative OB-fold domains that is common to all known SSB proteins of the *Deinococcus/Thermus* phylum. Figure 1 shows an alignment of *D. radiodurans*, *D. geothermalis*, *D. radiopugnans*, *D. grandis*, *D. proteolyticus*, *T. aquaticus* and *T. thermophilus* SSBs. Of these, the *Thermus* SSBs have been first characterized extensively biochemically and biophysically as a prototype for SSB proteins in homodimeric form, where each SSB monomer contains two OB-folds (Dąbrowski *et al.*, 2002a; 2002b). Two years ago SSB from *D. radiodurans* was also characterized (Bernstein *et al.*, 2004; Eggington *et al.*, 2004). Analysis shows that *Thermus/Deinococcus* SSBs are highly conserved. Protein sequence of *DgrSSB* shares 82% identity and 89% similarity to *D. radiodurans* R1 SSB, 44% identity and 63% similarity to *T. thermophilus* HB-8 SSB, 44% identity and 62% similarity to *T. thermophilus* VK-1 SSB and 45% identity and 63% similarity to *T. aquaticus* YT-1 SSB protein. *DprSSB* protein shares 69% identity and 82% similarity to *D. radiodurans* R1 SSB and 43%

identity and 63% similarity to *T. thermophilus* HB-8 SSB, 43% identity and 62% similarity to *T. thermophilus* VK-1 SSB and 43% identity and 61% similarity to *T. aquaticus* YT-1 SSB (Fig. 1). For both examined proteins the N-terminal fragment shares two, three times higher identity and similarity to adequate fragment of *D. radiodurans* R1 than to *E. coli* SSB protein. The C-terminal fragments of *DgrSSB* and *DprSSB* proteins are essentially more identical to *EcoSSB*. On the other hand, the N-terminal fragments of *DgrSSB* and *DprSSB* proteins do not share such a high degree of homology with *EcoSSB*. In the formation of homodimers, *Thermus/Deinococcus* SSBs maintain the bacterial trend of four OB folds per SSB protein oligomer. The structure of *DgrSSB* and *DprSSB* proteins is consistent with and reinforces the close phylogenetic relationship between *Deinococcus* and bacteria of the *Thermus* group of extremophiles.

Expression and purification of *DgrSSB* and *DprSSB*

Using the recombinant pBADD*grSSB* or pBADD*prSSB* plasmid, excellent inducible expression of a protein of the predicted size was obtained (Fig. 2, lane 1 and 1A). Both proteins were expressed in a soluble form in the cytosol. The recombinant proteins were purified by a heat denaturation and two column chromatography steps as described in Materials and Methods. There is some reduction of contamination by the host proteins after the heat treatment step (Fig. 2, lane 2 and 2A). These results were not surprising as we described previously that isolated SSB protein from mesophilic *D. radiopugnans* bacteria was highly thermostable (Filipkowski *et al.*, 2006b). The studied proteins also come from mesophilic microorganisms, for which the optimum temperature of growth is below 30°C (*D. grandis*). The applied overexpression system produces up to about 180 mg purified *DgrSSB* or *DprSSB* protein from 1 liter of induced culture. These proteins after concentration were purified with about 99% purity (Fig. 2, lane 5 and 5A).

Oligomerization status of *DgrSSB* and *DprSSB*

Molecular masses of *DgrSSB* and *DprSSB*, as calculated from the amino-acid sequences, are 32.29 kDa and 31.33 kDa, respectively. To determine the oligomeric state of these proteins, they were analyzed by gel filtration chromatography. The *DgrSSB* and *DprSSB* proteins eluted from a calibrated gel filtration column with estimated sizes of about 53.07 kDa and 61.06 kDa (not shown), consistent with homodimeric subunit composition in solution. This confirmed our prediction that *DgrSSB* and *DprSSB* exist in solution as a homodimers.



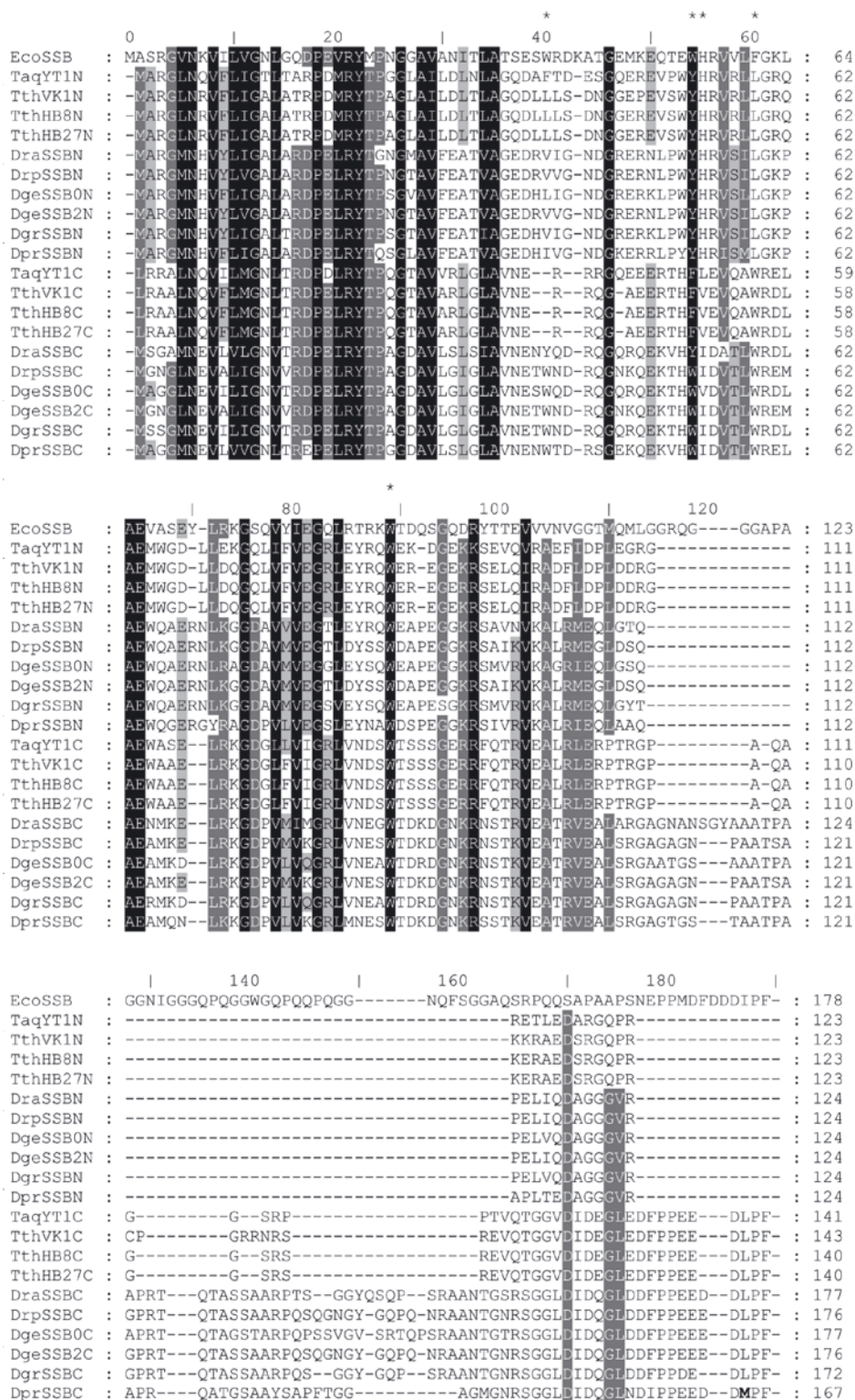


Figure 1. Multiple amino-acid sequence alignment of SSB-like proteins.

Alignment was performed by dividing amino-acids into six similarity groups: group 1, V, L, I and M; group 2, W, F and Y; group 3, E and D; group 4, K and R; group 5, Q and D; group 6, S and T. Description of similarity: white fonts on black boxes, 100% identity; white fonts on grey boxes, similarity <80%; black fonts on grey boxes, similarity <60%. Asterisks indicate conserved amino acids in the EcoSSB sequence that is engaged in stacking interaction with bases of ssDNA (W41, W55, F61 and W89) and stabilization of the tetramer (H56). Abbreviations: EcoSSB, *E. coli* K-12; TaqYT1, *T. aquaticus* strain YT-1; TthHB8, *T. thermophilus* strain HB-8; TthHB27, *T. thermophilus* strain HB-27; TthVK1, *T. thermophilus* strain VK-1; DraSSB, *D. radiodurans* strain R1; DrpSSB, *D. radiopugnans*; DgeSSB0, *D. geothermalis* strain 11300; DgeSSB2, *D. geothermalis* strain 11302; DgrSSB, *D. grandis*; DprSSB, *D. proteolyticus*; N, N-terminal ssDNA-binding domain; C, C-terminal ssDNA-binding domain.

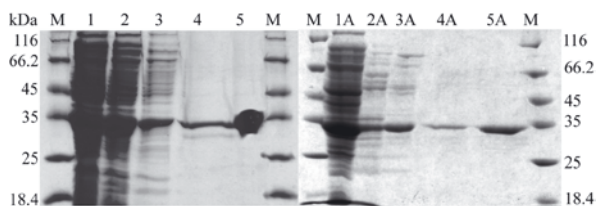


Figure 2. SDS-electrophoresis in 12% polyacrylamide gel of the fractions obtained by expression and purification of the recombinant *DgrSSB* from *E. coli* TOP10F⁺+pBAD-*DgrSSB* (left panel) and *DprSSB* from *E. coli* TOP10F⁺+pBAD-*DprSSB* ("A"-right panel).

Lane M, LMW markers (Fermentas), the molecular mass of proteins are marked; lane 1 and 1A, soluble protein cell extracts after arabinose induction of protein expression; lane 2, *DgrSSB* and 2A *DprSSB*, after heat treatment; lane 3 *DgrSSB* and 3A *DprSSB* after chromatography on QAE-cellulose column; lane 4 *DgrSSB* and 4A *DprSSB* after chromatography on ssDNA-cellulose column; lane 5 concentrated *DgrSSB* protein and 5A concentrated *DprSSB* protein.

DNA binding monitored by retardation assay

To determine the ability of *DgrSSB* and *DprSSB* proteins to bind to ssDNA, we carried out agarose gel mobility assays with fluorescein-labelled ss dT-oligonucleotides 35, 60, 70, 76 nucleotides in length (Fig. 3). For (dT)₃₅ and (dT)₆₀ there was a one complex regardless of protein concentration. A band of identical mobility was observed for (dT)₇₀ and (dT)₇₆ at low protein concentrations, but a second band with a lower mobility was observed at high protein concentrations. These results suggest that *DgrSSB* and *DprSSB* proteins bind to (dT)₃₅ and (dT)₆₀ as a single protein molecule whereas two SSB molecules bind to (dT)₇₀ and (dT)₇₆. The implications are that the minimum length of DNA that is required for efficient binding is between 30 and 35 nucleotides long.

DNA binding monitored by quenching of intrinsic tryptophan fluorescence

To explore the binding properties of examined SSB proteins further, we made use of fluorescence spectroscopy (Fig. 4). All homotetrameric and homodimeric SSB proteins studied so far show a dramatic decrease of tryptophan fluorescence when binding to ssDNA. With an excitation wavelength of 295 nm, the emission spectrum of SSB-like proteins at 25°C had a maximum at 348 nm, consistent with tryptophan fluorescence. On addition of a saturating quantity of ssDNA, the intrinsic fluorescence at 348 nm was quenched in 76% by *DgrSSB* and in 85% by *DprSSB*. The estimated size of ssDNA binding site in the presence of 2 mM and 100 mM of NaCl for *DgrSSB* and *DprSSB* proteins was about of 35±2nt. And practically binding-mode transition has not been observed when changing the ionic environ-

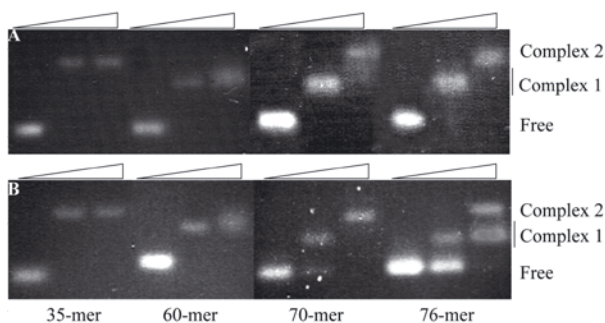


Figure 3. Binding of *DgrSSB* (A) and *DprSSB* (B) to single-stranded oligonucleotides – gel mobility shift assays.

ment from low (2 mM NaCl) to high salt (100 mM NaCl).

Thermostability

The half-lives of ssDNA-binding activity of *DgrSSB*, *DprSSB*, *DraSSB* and *TaqSSB* proteins at different temperatures and times were determined by gel mobility shift assay of poly(dT)₃₅ in 2% agarose gel (Fig. 5). The half-lives of ssDNA-binding activity of *DgrSSB* are 40 min at 55°C and 1 min at 65°C. *DprSSB* is only slightly more thermostable than *DgrSSB* with 90 min at 55°C and 1 min at 67.5°C. Previously described SSB proteins (*TaqSSB*, *DgeSSB* and *DrpSSB*) have higher thermostability than SSB proteins examined in this work (Dąbrowski *et al.*, 2002a; 2002b; Filipkowski *et al.*, 2006a; 2006b).

DgrSSB and *DprSSB* can replace *EcoSSB* *in vivo*

In the complementation experiments, we attempted to replace the resident plasmid (pRPZ146, *ori* ColE1, Tc^R, harboring a wild-type *ssb* gene) from *E. coli* RDP268(DE3) (Δ *ssb*::Kan) with the plasmids harboring test *ssb* genes (pET23D(+)*DgrSSB*, pET23D(+)*DprSSB* or pET23D(+)*EcoSSB*, *ori* ColE1,

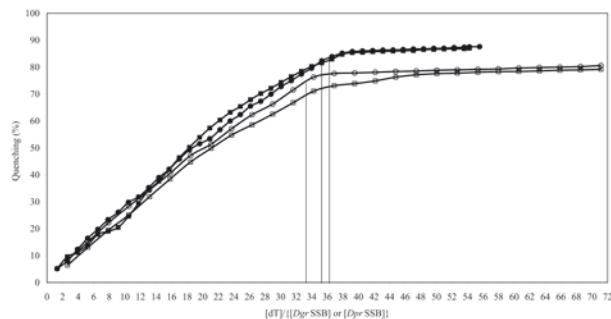


Figure 4. Inverse fluorescence titration of *DgrSSB* or *DprSSB* with (dT)₆₀.

A 1 nM sample of SSB protein (homodimer) was titrated with (dT)₆₀ at 2 mM (empty square) and 100 mM NaCl (empty circle) in standard buffer B and the same for *DprSSB* (filled figures).

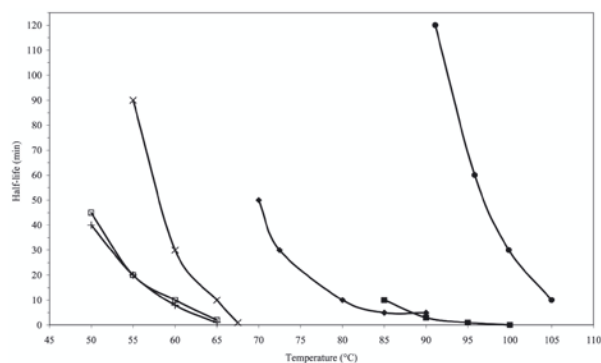


Figure 5. Half-lives of ssDNA-binding activity of *DraSSB* (□), *DgrSSB* (+), *DprSSB* (×), *DgeSSB* (◆), *TaqSSB* (■) and *DrpSSB* (●).

Ap^R). Since SSB is an essential protein, success in replacement of the original Tc^R plasmid by the incoming Ap^R plasmid, resulting in a Tc^S, Ap^R phenotype, shows that the test SSB complements the Δ ssb strain of *E. coli*. The pET23D(+)*EcoSSB* was used as a control to assess the efficacy of the complementation assay. After transformation of modified *E. coli* cells with pET23D(+)*DgrSSB*, pET23D(+)*DprSSB* or pET23D(+)*EcoSSB*, which encodes resistance to ampicillin, and subsequent inoculations, we could isolate clones that showed resistance to ampicillin and kanamycin but not to tetracycline. These clones must have lost the pRPZ146 plasmid encoding *EcoSSB*. Additionally, an analysis of plasmid DNA was carried out after plasmid isolation from tested strains. The results confirmed the presence of appropriate plasmids. Interestingly, under the same assay conditions, we obtained better growth of cultures containing pET23D(+)*DgrSSB* or pET23D(+)*DprSSB* than with pET23D(+)*EcoSSB* and the results were independent of IPTG induction of SSB expression.

DISCUSSION

In this article, we describe the identification, purification and characterization of SSB-like proteins from the mesophilic *D. grandis* and *D. proteolyticus* microorganisms. From sequence analysis, it is obvious that two ssDNA-binding domains in one mono-

mer of these proteins possess a canonical oligonucleotide binding fold, very similar to the homology observed in the structure of *D. radiodurans* SSB (Bernstein *et al.*, 2004). Gel filtration chromatography experiments of *DgrSSB* and *DprSSB* clearly showed that these proteins form dimers in solution.

We showed that the binding of *DgrSSB* or *DprSSB* to ssDNA results in a quench of tryptophan fluorescence at over 76% under all salt conditions used. The binding site size of *DgrSSB* or *DprSSB* is of about 35 nt per homodimer and is only slightly salt dependent. For *EcoSSB*, at least two distinctly different DNA-binding modes have been described (Lohman & Overman 1985). Whereas under high salt conditions, 65 nt bind per *EcoSSB* tetramer with almost 90% fluorescence quench, under low salt conditions 35 nt are sufficient to saturate the protein and quench its fluorescence by only 53%. The distinctly different binding mode of *EcoSSB* under high salt conditions could not be observed for *DgrSSB* and *DprSSB*, as for all known *Deinococcus/Thermus* SSBs. Based on the structure of a complex of *EcoSSB* and two molecules of (dC)₃₅, a model of the low salt binding mode of *EcoSSB* has been proposed (Raghuathan *et al.*, 2000). In this model, the Trp-54 residues of only two subunits of *EcoSSB* are involved in DNA binding. The most important difference between the *Deinococcus/Thermus* SSBs and *EcoSSB* lies in the fact that *Deinococcus/Thermus* SSBs contain two instead of one DNA-binding domain per monomer. *E. coli* SSB base-stacking residues are Trp-40, Trp-54, Phe-60, and Trp-88. The related residues in *DgrSSB* are Val-40, Tyr-53 (proceeds by Trp-52), Leu-59 and Trp-88 in the N-terminal OB domain. The same residues are present in *DprSSB* with only one exception: Tyr-53 is proceeds by Tyr-52. In the C-terminal OB domain there are Trp-164 (Tyr-164 in *DraSSB*), Trp-177 (Tyr-164 in *DraSSB*), Trp-183, and Trp-210 for both *DgrSSB* and *DprSSB*. Altogether, the C-terminal OB domain in the examined proteins contains homologous residues to the four aromatic base-stacking residues responsible for DNA binding by *EcoSSB* and only two of these residues are conserved in the N-terminal OB domain of the proteins (Fig. 1). A highly conserved His-55, important for homotetramerization of *EcoSSB*, is homologous to

Table 1. Percentages amino acid content of *EcoSSB*, *DraSSB*, *DrpSSB*, *DgeSSB0*, *DgeSSB2*, *DgrSSB* and *DprSSB* proteins.

SSB Protein	Ala	Arg	Asn	Asp	Cys	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val
<i>EcoSSB</i>	7.30	5.60	4.50	4.50	0.00	10.10	5.60	16.30	0.60	2.80	4.50	3.40	3.40	2.20	6.70	5.60	5.10	2.20	2.20	7.30
<i>DraSSB</i>	11.3	8.97	5.65	6.31	0.00	4.32	7.64	11.3	1.00	2.99	7.31	2.99	2.99	1.00	4.98	4.65	4.98	1.66	2.99	6.98
<i>DrpSSB</i>	10.33	8.00	6.33	6.67	0.00	3.67	7.67	12.33	1.00	2.33	7.33	4.33	2.67	1.00	5.33	4.33	5.00	2.33	2.00	7.33
<i>DgeSSB0</i>	10.96	9.30	3.32	6.31	0.00	4.65	7.64	11.63	1.33	1.99	8.64	2.99	1.99	1.33	5.65	4.98	5.65	2.33	1.33	7.97
<i>DgeSSB2</i>	10.33	8.00	6.33	6.67	0.00	3.67	7.67	12.33	1.00	2.33	7.33	4.33	2.67	1.00	5.33	4.33	5.00	2.33	2.00	7.33
<i>DgrSSB</i>	9.46	9.12	4.39	6.76	0.00	5.05	7.09	11.82	1.35	2.70	7.77	3.38	2.70	1.01	5.74	5.07	5.74	2.36	2.36	7.09
<i>DprSSB</i>	10.65	7.56	4.12	5.84	0.00	2.75	8.25	12.71	1.37	2.75	8.59	4.12	3.09	1.37	5.15	5.15	5.15	2.06	2.41	6.87

the corresponding His residue present only in the N-terminal domains of *DgrSSB* and *DprSSB* (as in other *Thermus/Deinococcus* SSBs). Two binding domains (N- and C-terminal OB-folds) could have evolved separately and amino acids known to be involved in DNA binding were conserved more in the C-terminal OB-fold than in the N-terminal one (see Fig. 1).

DgrSSB and *DprSSB*, like other SSB proteins, contain in the C-terminal part a proline- and glycine-rich region, which is expected to be very flexible. Similarly to the other homologs, in *DgrSSB* and *DprSSB* the proline- and glycine-rich region is followed by the last 10 amino acids, which are highly conserved in this class. For *EcoSSB*, it could be shown that this region is responsible for the interaction with other proteins involved in DNA metabolism (Kelman *et al.*, 1998; Genschel *et al.*, 2000; Handa *et al.*, 2001; Witte *et al.*, 2003). It has been speculated that the glycine- and proline-rich region acts as a spacer between the DNA-binding domain and the negatively charged region of the last 10 amino acids (Curth *et al.*, 1996). Thus, the formation of complexes between SSB proteins and their interaction partners could be facilitated by an easy access of this region.

EcoSSB is essential for the survival of the *E. coli* cell (Porter *et al.*, 1990). Using an *E. coli* *ssb* mutant strain, we could show that *DgrSSB* and *DprSSB* can take over the function of *EcoSSB* *in vivo*. Since *DgrSSB* or *DprSSB* (like as other SSBs from *Deinococcus/Thermus* phylum) as dimers contain only two instead of four C-termini, two C-termini seem to suffice for the *in vivo* function of SSB. It has been speculated that the loss of two of the four C-termini in *DraSSB* may lead to functional differences (Eggington *et al.*, 2004). Our data clearly show that such functional differences, even if they exist, do not play an important role in the function of SSB *in vivo*.

Our observations revealed that *DgrSSB* and *DprSSB* (like other known *Deinococcus/Thermus* SSBs) seem to be a normal representatives of bacterial SSB proteins. Except for the absence of salt binding-mode transition, *Deinococcus/Thermus* SSBs have all the essential properties of the prototype *EcoSSB*, including the ability to take over the vital role of *EcoSSB* in *E. coli* cells. The question as to why evolution chose to create homodimeric bacterial SSB proteins remains open.

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