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Identification and characterization of single-stranded DNA-binding protein from the facultative psychrophilic bacteria *Pseudoalteromonas haloplanktis*

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ABSTRACT

Single-stranded DNA-binding protein (SSB) plays an important role in DNA metabolism such as DNA replication, repair, and recombination, and is essential for cell survival. This study reports on the *ssb*-like gene cloning, gene expression and characterization of a single-stranded DNA-binding protein of *Pseudoalteromonas haloplanktis* (*Pha*SSB) and is the first report of such a protein from psychrophilic microorganism. *Pha*SSB possesses a high sequence similarity to *Escherichia coli* SSB (48% identity and 57% similarity) and has the longest amino acid sequence (244 amino acid residues) of all the known bacterial SSBs with one OB-fold per monomer. An analysis of purified *Pha*SSB by means of chemical cross-linking experiments, sedimentation analysis and size exclusion chromatography revealed a stable tetramer in solution. Using EMSA, we characterized the stoichiometry of *Pha*SSB complexed with a series of ssDNA homopolymers, and the size of the binding site was determined as being approximately 35 nucleotides long. In fluorescence titrations, the occluded site size of *Pha*SSB on poly(dT) is 34 nucleotides per tetramer under low-salt conditions (2 mM NaCl), but increases to 54–64 nucleotides at higher-salt conditions (100–300 mM NaCl). This suggests that *Pha*SSB undergoes a transition between ssDNA binding modes, which is observed for *Eco*SSB. The binding properties of *Pha*SSB investigated using SPR technology revealed that the affinity of *Pha*SSB to ssDNA is typical of SSB proteins. The only difference in the binding mode of *Pha*SSB to ssDNA is a faster association phase, when compared to *Eco*SSB, though compensated by faster dissociation rate. When analyzed by differential scanning calorimetry (DSC), the melting temperature (T_m) was determined as 63 °C, which is only a few degrees lower than for *Eco*SSB.

1. Introduction

Psychrophilic microorganisms have been relatively poorly studied, although they inhabit a much larger part of our planet, for instance, oceans, than their mesophilic or thermophilic counterparts do. Proteins isolated from psychrophilic microorganisms possess special properties, such as the ability to be active even at temperatures below 0 °C, which offers novel opportunities for biotechnological exploitation. Recently, research on psychrophilic proteins has intensified and considerable progress has been made in the characterization of the molecular basis of their physicochemical features. Although the molecular basis of cold-adaptation is not yet fully understood, the results of numerous studies suggest a consensus pertaining to subtle changes that synergistically act to fine-tune the molecular structure of psychrophilic proteins in order to shift their thermal optimum, unlike their mesophilic homologs. It is noteworthy, however, that the cold adaptation strategies appear to have evolved independently in various protein families and can vary greatly between different proteins. In this study, the characterization of single-stranded DNA-binding protein (SSB) from psychrophilic bacteria is presented for the first time.

Single-stranded DNA-binding proteins are indispensable elements in the cells of all living organisms. SSB proteins interact with ssDNA in a manner independent of sequence, preventing them both from forming secondary structures and from degradation by nucleases (Greipel et al., 1989). In this way, SSB-binding proteins participate in every process involving ssDNA, such as, for instance, replication, repair and recombination (Alani et al., 1992; Lohman and Overman, 1985; Meyer and Laine, 1990; Shereda et al., 2008). Based on oligomeric structure, SSBs can be classified into four groups: monomeric, homodimeric, heterotrimeric and homotetrameric. Although there are differences in amino acid sequences, SSBs have a high-conservative domain, the oligonucleotide/oligosaccharide-binding fold, referred to as the

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OB-fold, which is responsible for binding with ssDNA (Murzin, 1993). In the bacterial single-stranded DNA-binding proteins described so far, four OB-fold domains form an active protein. SSbs are present in all three branches of live organisms and in viruses, performing similar functions but displaying little sequence similarity and very different ssDNA binding properties.

In this article, the purification and characterization of the SSB protein from the psychrophilic bacteria *Pseudoalteromonas haloplanktis* is described. The microorganism has been isolated from an Antarctic coastal sea water sample collected in the vicinity of the French Antarctic station Dumont d'Urville, Terre Adélie. These aerobic bacteria belong to the gamma class of *Proteobacteria* phylum (Médigue et al. 2005). The representatives of this species grow in a temperature range of between 4 and 25 °C with an optimum of 15 °C (Papa et al., 2009). The cells are well adapted to salt, and although they can grow in low osmolarity media, optimal growth is between 1.5 and 3.5% NaCl (Médigue et al., 2005). *P. haloplanktis* TAC125 was used for the construction of a novel genetic system for the production and secretion of recombinant proteins at low temperature (Cusano et al., 2006). This system allows the production of 'difficult' proteins, which are not successfully expressed in any other expression systems (Cusano et al., 2006; Parrilli et al., 2008). *P. haloplanktis* is also recognized as an important resource of bioactive compounds and cold-adapted proteins, including enzymes (e.g. Hayashida-Soiza et al., 2008; Mitova et al., 2005). The *P. haloplanktis* TAC125 genome consists of two chromosomes with a base composition of 41 and 39.3 mol% guanine + cytosine (Médigue et al., 2005). On the basis of the *ssb* gene organization and the number of *ssb* genes paralogs, bacteria are classified in four different groups (Lindner et al., 2004). *P. haloplanktis* was classified as group III, which contains bacteria with *ssb* gene organization *uvrA-ssb*.

The aim of this study was to clone and overexpress a *P. haloplanktis ssb*-like gene in *E. coli*, purify the gene product and study the biochemical properties.

2. Materials and methods

2.1. Bacterial strains, plasmids, enzymes and reagents

P. haloplanktis TAC 125 (CIP 108707) strain was purchased from CIP (Collection de l'Institut Pasteur Paris, France). *E. coli* TOP10 (Invitrogen, USA) was used for the genetic constructions and gene expression. pBAD/*myc*-HisA plasmid (Invitrogen, USA) was used for constructing the expression system. The reagents for PCR were obtained from Blirt SA - DNA-Gdańsk (Poland). Specific primers, oligodeoxynucleotides and oligonucleotides 5′-end-labeled with fluorescein were purchased from Sigma (USA). The restriction enzymes were purchased from NEB (USA). *Eco*SSB and *Tma*SSB were produced and purified in our laboratory in line with published procedure (Dąbrowski and Kur 1999; Dąbrowski et al. 2002a, 2002b; Olszewski et al. 2010).

2.2. Cloning of the ssb-like gene from Pseudoalteromonas haloplanktis

DNA from *P. haloplanktis* TAC 125 was isolated using an ExtractMe DNA Bacteria Kit (Blirt SA - DNA-Gdańsk, Poland). The specific primers for PCR amplification were designed and synthesized on the basis of the known *ssb*-like gene sequence (Gene Bank Accession No. NC007481) from *P. haloplanktis* TAC 125. The forward primer was phassbNde, 5′-TTA CAT **ATG GCA CGC GGT GTG AAC AAA GTA AT** (32 nt, containing a *Nde*I recognition site) and reverse primer, phassbHind, 5′-TTA AAG CTT ***TCA* GAA CGG TAT GTC GTC GTC AAA ATC** (36 nt, containing a *Hin*dIII recognition site and UGA stop codon, italicized). The boldface parts of the primer sequences are complementary to the nucleotide sequence of the *ssb*-like gene of *P. haloplanktis* TAC 125, whereas the 5′ overhanging ends of primers contain recognition sites for restriction endonucleases (underlined) and are designed to facilitate cloning. The PCR reaction solution consisted of 0.2 μg of *P. haloplanktis* TAC 125 genome DNA, 1 μl (10 μM) of each primer, 2.5 μl (10 mM) dNTPs, 1 μl (25 mM) $MgCl_2$, 2.5 μl of 10 x PCR buffer (200 mM Tris–HCl, pH 8.8, 100 mM KCl, 100 mM $(NH_4)_2SO_4$, 1% Triton X-100) and 2U *Pwo* DNA polymerase (Blirt SA DNA-Gdańsk, Poland). 35 cycles were performed, using the Veriti® 96 Well Thermal Cycler (Applied Biosystems, USA), with a temperature profile of 60 s at 94 °C, 60 s at 50 °C and 60 s at 72 °C. The amplification products were analyzed by electrophoresis on 1% agarose gel stained with ethidium bromide, at a final concentration of 0.5 μg/ml. Specific, approximately 750 bp, PCR products were obtained and purified, using the ExtractMe Gel-Out Kit (Blirt SA DNA-Gdańsk, Poland). The PCR products was digested with *Nde*I and *Hin*dIII (NEB, USA), then purified, using an ExtractMe Clean-Up Kit (Blirt SA DNA-Gdańsk, Poland) and ligated into pBAD/*myc*-HisA plasmid (Invitrogen, USA) between the *Nde*I and *Hin*dIII sites. The *E. coli* TOP10 cells were transformed with the ligation mixture and 16 colonies were examined for the presence of the *ssb*-like gene from *P. haloplanktis* TAC 125, using a gel retardation assay and restriction analysis. One clone was selected and sequenced to confirm the presence of that gene.

2.3. Protein sequence analysis

The amino acid sequence of *PhaSSB* was analyzed using standard protein–protein BLAST and RPS-BLAST. Multiple sequence alignment was generated in ClustalX, using a PAM 500 scoring matrix. The results were prepared using the GeneDoc editor program (www.psc.edu/biomed/genedoc).

2.4. Expression and purification of PhaSSB

The *E. coli* TOP10 strain transformed with pBAD/*Pha*SSB was grown at 30 °C in Luria-Bertani medium, supplemented with 100 μg/ml of ampicillin, to an OD_{600} of 0.3, and was induced by incubation in the presence of arabinose, at a final concentration of 0.02%, for 18 h. The cells were then harvested by centrifugation at 4612 × *g* for 15 min and the pellets were resuspended in 30 ml of buffer A (20 mM Tris–HCl pH 8.0, 50 mM NaCl, 1 mM EDTA pH 8.0, 0.1% Triton X-100). The samples were sonicated six times, for 30 s at 4 °C, and centrifuged at 10,000 × *g* for 25 min. The clarified supernatant was then applied directly onto a QAE-cellulose column (50 ml bed volume, EMD, USA) preequilibrated with 4 vol buffer B (20 mM Tris–HCl pH 8.0, 50 mM NaCl, 1 mM EDTA pH 8.0). The SSB-like protein was eluted with a linear gradient of 0.05–2 M NaCl in buffer B. The SSB-containing fractions were detected by SDS-PAGE electrophoresis, after which, they were combined and loaded onto a ssDNA-cellulose column (5 ml, USB, USA) equilibrated with buffer C (20 mM Tris–HCl pH 8.0, 0.25 M NaCl, 1 mM EDTA pH 8.0). The *Pha*SSB was eluted with 1.5 M NaCl and 50% ethylene glycol. The elution fraction was dialyzed against D buffer (20 mM Tris–HCl pH 8.0, 0.5 M NaCl) and concentrated to 2 mg/ml, using the Amicon Ultra-15 Filter Device MWCO 10000 (Millipore, USA). The purity of the *Pha*SSB was estimated using SDS-PAGE and the quantities were examined spectrophotometrically using the extinction coefficient $\varepsilon_{280} = 1.04 \times 10^5\ M^{-1}$ (tetramer) cm^{-1}.

2.5. Estimation of the native molecular mass

The native molecular mass of *Pha*SSB was determined by three independent methods: (i) chemical cross-linking experiments, (ii) sedimentation in a 15–30% glycerol gradient and (iii) size exclusion chromatography.

Chemical cross-linking experiments were carried out using 0.5, 1 and 1.5% (v/v) glutaraldehyde for 1–20 min, with 1 and 4 nmol of *Pha*SSB, at 25 °C. The reaction was quenched by the addition of 1 M Tris–HCl (pH 8.0), and the cross-linked protein solutions were then analyzed using SDS-PAGE (12%).

For ultracentrifugation linear 15–30% (w/v) glycerol gradients, containing loading buffer (50 mM Tris–HCl, pH 7.5, 0.5 M NaCl, 1 mM EDTA and 5 mM β-mercaptoethanol), were prepared in 5 ml Beckman centrifuge tubes. 50 μl of a 300 μM *Pha*SSB protein in loading buffer and the corresponding amounts of *Eco*SSB and standard proteins (carbon anhydrase 29 kDa, bovine albumin 66 kDa, alcohol dehydrogenase 150 kDa, β-amylase 200 kDa) were layered over 3.5 ml of the glycerol gradient and were centrifuged in individual tubes. The gradients were centrifuged at 4 °C in a Beckman SW 60 rotor at 46,000 rpm for 24 h; the fractions were then collected from the top. The proteins present in the fractions were separated by SDS-PAGE.

2.6. Size exclusion chromatography of PhaSSB was carried out on a Superdex HR 200 column

(Amersham Bioscience AB, Sweden). The elution pattern of *Pha*SSB protein was then compared with those of standard proteins: thyroglobulin (669 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa).

2.7. Agarose gel electrophoresis mobility shift assays (EMSA)

A fixed quantity (10 pmol) of 5′-end fluorescein-labeled oligonucleotides $(dT)_{35}$, $(dT)_{76}$ and $(dT)_{120}$ were incubated with 25, 50, 100, 200, 400 and 800 pmol of *Pha*SSB in a binding buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl and 1 mM EDTA) for 10 min, at 25 °C, to a final reaction volume of 20 μl. The reaction products with oligos were then loaded onto 2% agarose gel, either with or without ethidium bromide, and separated by electrophoresis in a TAE buffer (40 mM Tris acetate pH 7.5 and 1 mM EDTA).

A ssDNA of phage M13 (0.05 pmol) was incubated with 2, 8, 16, 32, 60, 70, 80 and 100 pmol of *Pha*SSB in a binding buffer (20 mM Tris–HCl pH 8.0, 100 mM NaCl and 1 mM EDTA) for 10 min, at 25 °C, to a final reaction volume of 20 μl. The reaction products were then loaded onto 0.5% agarose gel, either with or without ethidium bromide, and separated by electrophoresis in a TAE buffer (40 mM Tris acetate pH 7.5 and 1 mM EDTA).

The bands corresponding to the unbound ssDNA and various *Pha*SSB-ssDNA complexes were visualized under UV light and photographed.

2.8. Fluorescence titration

Fluorescence titrations were carried out in a Perkin-Elmer LS-5B luminescence spectrometer, as described earlier (Curth et al., 1993). The binding reactions were assembled in 2 ml buffer of 20 mM Tris–HCl pH 8.0, 1 mM EDTA containing 2 mM, 100 mM or 300 mM NaCl and incubated at 25 °C. A fixed quantity of *Pha*SSB was incubated in the appropriate buffer at 25 °C with increasing quantities of $(dT)_{250}$ oligonucleotide at excitation and emission wavelengths of 295 and 348 nm, respectively. Binding curve analyses were carried out using Schwarz and Watanabe's model (Schwarz and Watanabe, 1983).

2.9. Surface plasmon resonance (SPR) measurements

Biacore 3000 was used at a temperature of 25 °C for the SPR measurements. The streptavidin coated chip (SA chip, GE Healthcare, USA) was first washed with 10 mM NaOH, 1 M NaCl to remove loose streptavidin. Biotinylated ssDNA 60-mer (5′-AATTCTGGGTGTGTGGGTGTGTGGGTGTGTGGGTGTGGTCAAGTTG-ACTACGTATACATC-biotin-3′) was bound at a level of 500 RU at channel number 2, leaving flow cell number 1 as a reference. Different concentrations of *Pha*SSB in a HBS buffer (10 mM Hepes, pH 7.4, 150 mM NaCl and 0.005% P20) were injected onto the chip, with a flow rate of 30 μl/min. Between injections, the flow cells were regenerated with 0.01% SDS. As a control experiment, the interaction between recombinant *Eco*SSB and ssDNA 60-mer was also studied, in line with the protocol described for *Pha*SSB. The binding parameters for all settings were calculated with BiaEval 3.0 software.

2.10. Melting point destabilization of dsDNA

Melting point curves were obtained by measuring the change in A_{260} in a Cary300Bio UV–Visible spectrophotometer (Varian) in 20 mM sodium phosphate buffer pH 7.5 containing 0.1 M NaCl and 1 mM EDTA (Augustyns et al., 1991). A mixture of dsDNA and the *Pha*SSB (or *Eco*SSB, or *Tma*SSB) was gradually heated from 25 to 95 °C with heating rate of 1 °C/min. The assay was performed using duplex DNA (44 bp) composed of two oligonucleotides: 5′-GAA CCG GAG GAA TGA TGA TGA TGA TGG TGC GGT TTG TCG GAC GG-3′ and 5′-CCG TCC GAC AAA CCG CAC CAT CAT CAT CAT CAT TCC TCC GGT TC-3′.

2.11. Thermostability

Microcalorimetric measurements were performed using a NanoDSC microcalorimeter (Calorimetry Science Corporation, USA). Samples containing approximately 2.0 mg/ml SSB (*Pha*SSB or *Eco*SSB or *Tma*SSB) in 50 mM of potassium phosphate buffer with a pH of 7.5 and 150 mM of NaCl were analyzed. The calorimetric scans were carried out between 0 and 100 °C, with a scan rate of 1 °C/min. The reversibility of the transition was checked by cooling and reheating the same sample with a scan rate of 1 °C/min. Association phase corresponds to 60 s of injections, dissociation can be observed between 65 and 170 s of experiment. The results from the DSC measurements were analyzed with NanoAnalyze Software V 1.1 (TA Instruments, USA).

3. Results

3.1. PhaSSB sequence analysis

The sequence analysis of the *P. haloplanktis* genome indicated the presence of a single *ssb*-like gene. On the basis of this nucleotide sequence, the *Pha*SSB protein contains 244 amino acid residues including the N-terminal methionine and has a predicted molecular weight of 26.2 kDa. The RPS-BLAST analysis of the primary structure revealed the presence of two distinctive regions: one putative OB-fold domain (from amino acid 1–106) and one C-terminal domain containing five conserved DDIPF terminal amino acid residues, which are common in all known bacterial SSB proteins. It has the longest sequence among the SSBs described to date with one OB-fold domain per monomer and with the extra residues in the intrinsically disordered C-terminal domain.

Fig. 1 shows the multiple amino acid alignment of the SSB proteins from the psychrophilic *P. haloplanktis* and *Shewanella woodyi*, mesophilic *Escherichia coli* and *Bacillus subtilis*, and thermophilic *Thermoanerobacter tengcongensis* and *Thermotoga maritima* microorganisms; all the said proteins contain one OB fold domain per monomer. The protein sequence of *Pha*SSB shares 48% identity and 57% similarity to the *E. coli* K12 SSB, 45% identity and 55% similarity to the *Shewanella woodyi* SSB, 24% identity and 37% similarity to the *B. subtilis* SSB, 18% identity and 30% similarity to

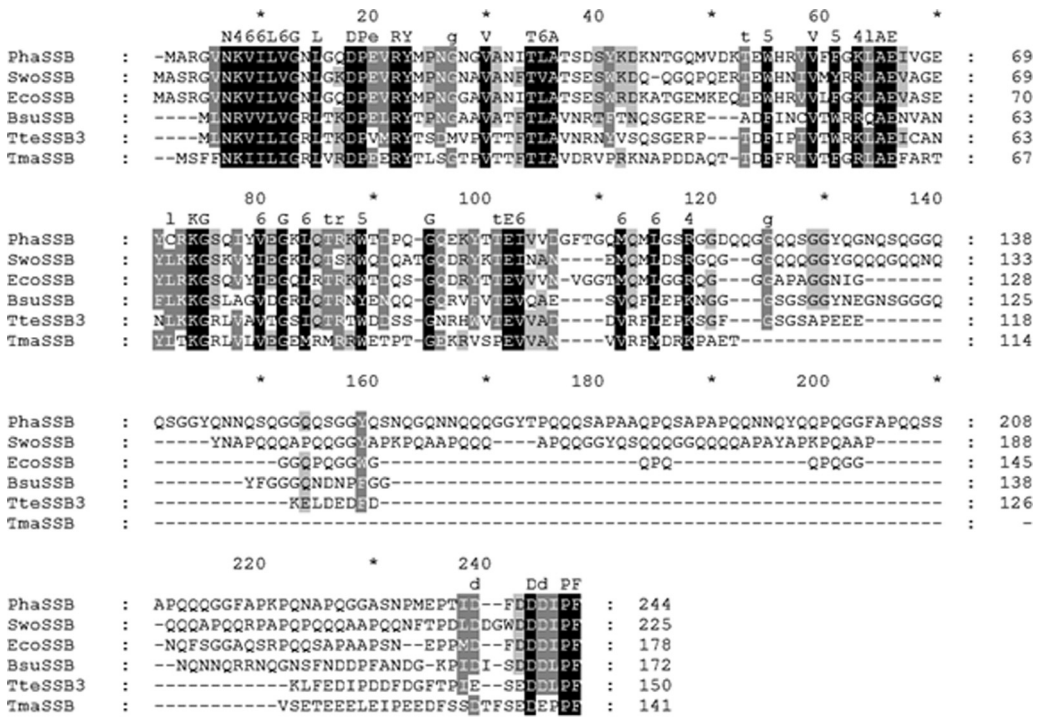

Fig. 1. The multiple amino acid alignment of PhaSSB with SSBs from psychrophilic, mesophilic and thermophilic bacteria. The alignments were performed by dividing the amino acids into six similarity groups: group 1 V, L, I, M; group 2 W, F, Y; group 3 E, D; group 4 K, R; group 5 Q, D; and group 6 S, T. The capital letters represent single amino acid codes. White fonts on black represent 100% similarity, white fonts on gray denote <80% similarity, and black fonts on gray show <60% similarity. PhaSSB *Pseudoalteromonas haloplanktis* TAC 125 (NC007481), SwoSSB *Shewanella woodyi* (NC010506), EcoSSB *Escherichia coli* K12 (NZGL883800), BsuSSB *Bacillus subtilis* (NC000964), TteSSB3 *Thermoanerobacter tengcongensis* MB4 (NC003869), and TmaSSB *Thermotoga maritima* MSB8 (NC000853).

the *Thermoanaerobacter tengcongensis* SSB3, and 15% identity and 28% similarity to the *Thermotoga maritima*. The homology between these proteins refers mainly to the N-terminal domain and five terminal amino acids of the C-terminal domain.

There are some features commonly observed in psychrophilic proteins, as compared to their mesophilic equivalents, including a lower Pro content, which comes from Pro deletion or substitution by other small residues such as, for example, Ala; a higher Gly content; a lower basic amino acid content, particularly Arg (decrease Arg/(Arg + Lys) ratio); fewer hydrogen bonds and aromatic interactions; and more polar, and less hydrophobic, residues (destabilization of the hydrophobic core). All these features act synergistically to increase the number of degrees of conformational freedom by introducing flexible residues on the protein surface and destabilizing the protein core by weakening the intramolecular forces. In this context, the *Pha*SSB protein has some cold adaptation features.

The *Pha*SSB protein has a charged (Asp, Glu, Lys and Arg; 14.4%) residues content which is lower than that of *Eco*SSB (19.1%) and much lower than the SSBs from thermophilic bacteria (*Tte*SSB3–30.0% and *Tma*SSB–33.3%) (Table 2). These residues in the thermophilic proteins may be involved in the stabilization of the interdomain surface by means of ionic networks. The content of Arg residues (2.5%) and the ratio of Arg/(Arg + Lys) (0.38) are definitely lower in the psychrophilic *Pha*SSB than in their mesophilic *E. coli* equivalent (5.6% and 0.62, respectively) and thermophilic SSBs (*Tte*SSB3–6.0%, 0.53 and *Tma*SSB–10.6%, 0.75. This feature has been considered as a hallmark of psychrozymes (Feller and Gerday, 1997; Feller et al., 1991, 2003; Gerday et al., 1994, 1997, 2000; Russell, 2000). Arginine is normally capable of forming multiple salt bridges with acidic (Asp and/or Glu) amino acid residues and hydrogen bonds with other amino acids. Thus the decrease of Arg content, even the conservative replacement of Arg with Lys, leads to a reduction in the number of salt bridges.

Psychrophilic *Pha*SSB (15.6%) and mesophilic *Eco*SSB (16.3%) possess a higher content of glycine than thermophilic SSBs (6.0% and 4.3% for *Tte*SSB3 and *Tma*SSB, respectively). This accords with the known tendency for thermostable proteins to have a preference for a decrease in the Gly content in positions of low structural importance for fold conservation (Matthews et al., 1987; Korolev et al., 1995).

The *Pha*SSB is high in glutamine and asparagine residues; at 25.5%, the content is nearly twice that of *Eco*SSB (14.6%) and much higher than for thermophilic SSBs (5.3 and 2.8% for *Tte*SSB3 and *Tma*SSB, respectively). 40 of the 46 glutamine residues are located in the C-terminal fragment of *Pha*SSB, which is 29% of this domain.

The *Pha*SSB turned out to have 40.2% of polar amino acid residues (N, S, Y, T, Q). This is considerably more than that found in the mesophilic *Eco*SSB (27.4% of all residues) and far greater than for the thermophilic SSBs (21.3 and 19.8% for *Tte*SSB3 and *Tma*SSB, respectively). The ratio of polar to non-polar amino acid residues is known to be one of the major determinants of protein stability, as is the fact that increasing the fraction of polar and charged residues leads to protein disorder (Gerday et al., 1997). In particular, as Russell (2000) and Zuber (1988) pointed out, psychrophilic proteins appear to have more polar residues than thermophiles or mesophiles.

Table 1
Binding parameters for the *Pha*SSB interaction with single-stranded DNA. The kinetic data of binding the *Pha*SSB protein (analyte) to the 60-mer ssDNA (ligand) were fitted using the Langmuir binding model. The parameters calculated for the *Eco*SSB are based on the control experiment conceived as part of the study. The data shown are the means of at least three independent measurements.

	K_D [M]	k_{on} [1/Ms]	k_{off} [1/s]
*Pha*SSB versus ssDNA	$9.4 \times 10^{-9} \pm 4.51$	$3.77 \times 10^{5} \pm 1.76$	$2.94 \times 10^{-3} \pm 0.27$
*Eco*SSB versus ssDNA	$5.36 \times 10^{-9} \pm 1.08$	$3.45 \times 10^{4} \pm 0.83$	$1.85 \times 10^{-4} \pm 0.23$

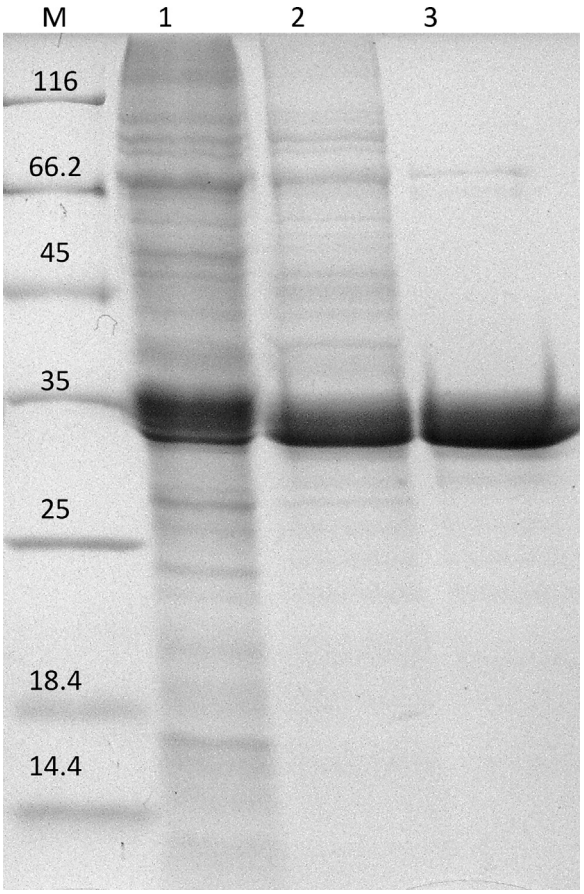

Fig. 2. The expression and purification of *Pha*SSB. The proteins were analyzed on a 12% polyacrylamide gel. Lane M: Unstained Protein Weight Marker (Fermentas, Lithuania), with the molecular mass of the proteins marked. Lane 1: soluble protein cell extracts after arabinose induction of protein expression (20 μl). Lane 2: *Pha*SSB after chromatography on QAE-cellulose column (20 μl). Lane 3: *Pha*SSB after chromatography on ssDNA-cellulose column (10 μl).

3.2. The cloning, expression and purification of PhaSSB

Using the pBAD*Pha*SSB recombinant plasmid, an arabinose-inducible expression system for *Pha*SSB was produced. The protein was expressed in soluble form in cytosol. The *E. coli* overexpression system used in this study produced approximately 30 mg of purified *Pha*SSB protein from 1 L of induced culture. The purity of the protein preparation was around 98% (Fig. 2). It should be noted an abnormal electrophoretic mobility of purified *Pha*SSB at a higher molecular weight (approximately 35 kDa) than the expected theoretical molecular weight (26.2 kDa). Mass spectroscopy analysis of the purified subunit confirmed the expected theoretical molecular weight and amino acid sequence of *Pha*SSB (results not shown).

3.3. The oligomerization status of PhaSSB

In chemical cross-linking experiment using glutaraldehyde, the *Pha*SSB complexes were found at a position corresponding to a molecular mass of around 130–150 kDa (Fig. 3A). This suggested that *Pha*SSB forms a homotetramer in solution.

The oligomeric status of *Pha*SSB was analyzed by centrifugation in 15–30% (w/v) glycerol gradients. To prevent nonspecific aggregation of the protein during experiments, NaCl at a final concentration of 0.5 M was added to the solutions used for the glycerol gradients. We observed that *Pha*SSB migrates more slowly in SDS-PAGE gels than would be expected on the basis of its predicted molecular mass. The SSB from *P. haloplanktis* was found at a position

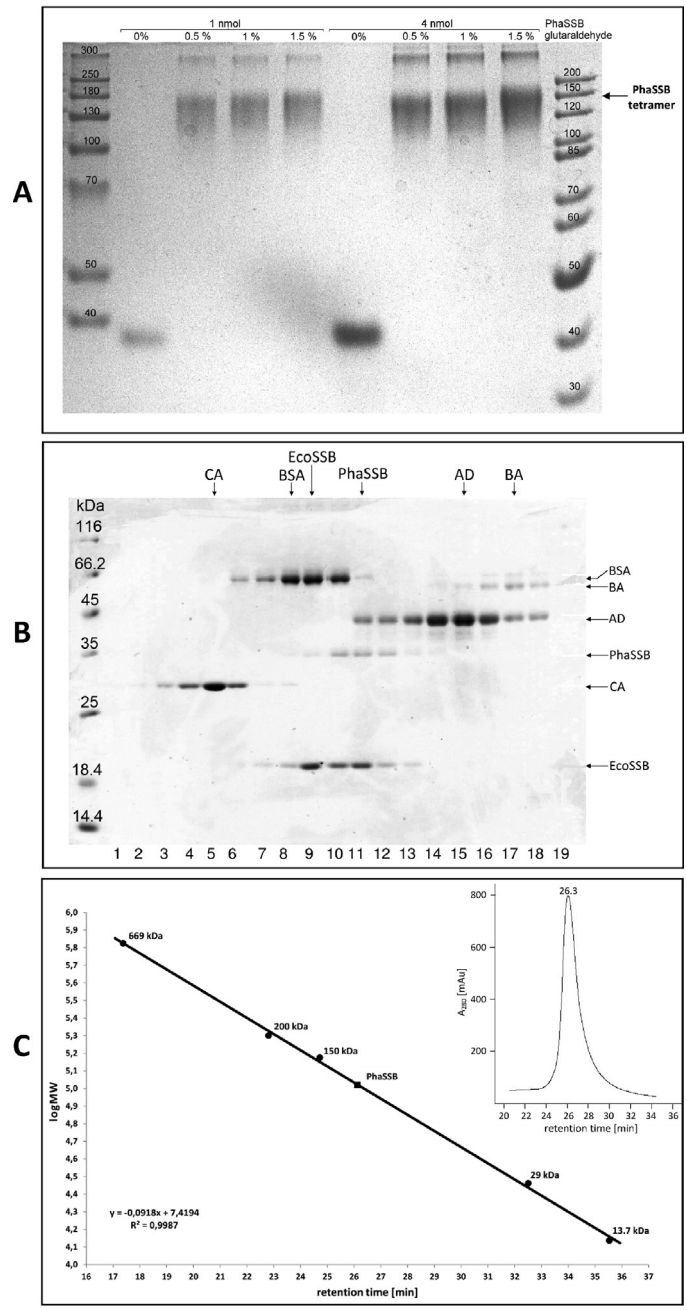

Fig. 3. Estimation of the native molecular mass of *Pha*SSB. (A) The results of chemical cross-linking experiments using 0.5, 1 and 1.5% (v/v) glutaraldehyde for 1–20 min with 1 and 4 nmol of *Pha*SSB at 25 °C. The fractions were analyzed using SDS-PAGE. The highest concentration of PhaSSB tetramer determined densitometrically is indicated by arrow (about 140 kDa). (B) Sedimentation analysis of *Pha*SSB, *Eco*SSB and standard proteins. Lane M: Unstained Protein Weight Marker (Fermentas, Lithuania), with the molecular mass of the proteins marked. 50 μl of 300 μM *Pha*SSB and the corresponding quantities of *Eco*SSB and standard proteins were centrifuged in linear 15–30% (w/v) glycerol gradients. The gradient was fractionated from the top into 19 fractions, and 5-μl portions from fractions 1 to 19 were analyzed by SDS-PAGE followed by Coomassie blue staining. The fractions at which the maximal amount of protein appears are indicated with arrows. The standard proteins used are CA, carbonic anhydrase (29 kDa); BSA, bovine serum albumin (66 kDa) and AD, alcohol dehydrogenase (150 kDa, tetramer); BA, β-amylase (200 kDa, tetramer). (C) Size exclusion chromatography of *Pha*SSB on Superdex HR 200 column. A standard linear regression curve was generated by plotting the log of the molecular mass of the calibration proteins against their retention times (min) and is shown. The calibration proteins include: thyroglobulin (669 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa).

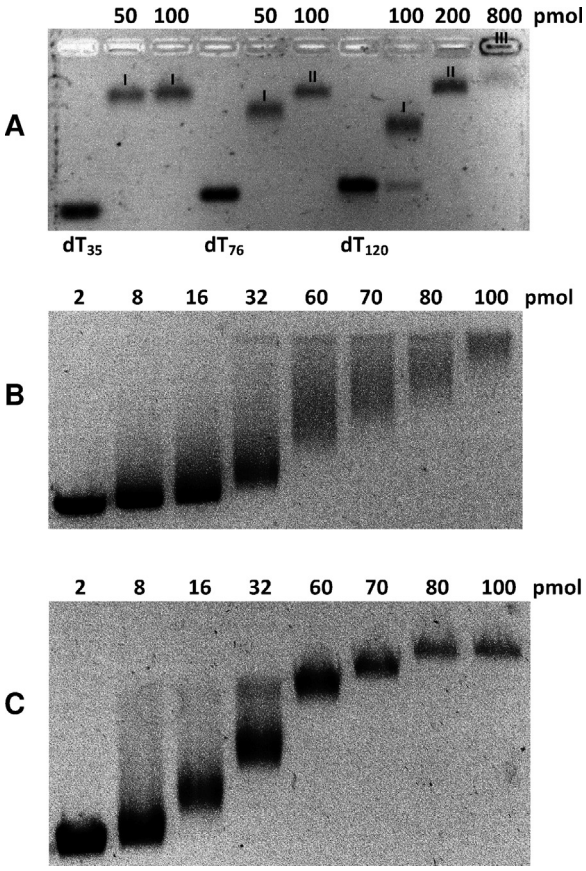

Fig. 4. Binding of *Pha*SSB to oligo(dT) and to M13 ssDNA. (A) Binding of *Pha*SSB to a fixed quantity (10 pmol) of oligonucleotodes dT_{35}, dT_{76} and dT_{120}. Symbols I, II and III describe *Pha*SSB:dT complexes. The concentration of *Pha*SSB in each lane is shown on the top of the gel. Binding of *Pha*SSB to M13 ssDNA at 2 mM NaCl (B) and at 300 mM of NaCl (C). The samples contain a fixed quantity of M13 ssDNA (0.05 pmol) and an increasing concentration of *Pha*SSB; the latter is shown on the top of the gel.

corresponding to a molecular mass of approximately 35 kDa (Fig. 3B), while its calculated molecular mass is 26.2 kDa. The centrifugation in glycerol gradients of *Pha*SSB was carried out three times and the same sedimentation behaviors were observed in all the independent tests. The sedimentation patterns of *Pha*SSB, *Eco*SSB and standard proteins in the glycerol gradients confirmed our prediction that *Pha*SSB exists as a homotetramer in solution.

Analysis of purified *Pha*SSB by size exclusion chromatography revealed single peak with a molecular mass of about 105 kDa (Fig. 3C). This value is 4 times the molecular weight of a *Pha*SSB monomer. This again confirmed our prediction that *Pha*SSB protein exists as a homotetramer.

3.4. ssDNA-binding properties

The purified *Pha*SSB protein was analyzed for single-stranded DNA binding activity. In these experiments, a fixed concentration of $(dT)_n$ (n = 35, 76 or 120 nucleotides in length) were incubated with various concentrations of *Pha*SSB and the resulting complexes were analyzed using agarose gel electrophoresis (Fig. 4A). When the dT_{35} was incubated with increasing concentrations of the SSB protein, a single band of reduced mobility, which remained constant even at higher protein concentrations (complex I), was observed. A band of analogical mobility was observed for the $(dT)_{76}$ at low protein concentrations, but a second band with a lower mobility was observed at high protein concentrations (complex II). When the *Pha*SSB:dT_{120} complexes were analyzed, a third band with a lower mobility was detected (complex III). The implications are that the length of ssDNA that is required for efficient protein binding is approximately 35 nucleotides.

The binding of the *Pha*SSB protein to the naturally occurring circular M13 ssDNA (6429 b) was also examined. A fixed quantity of M13 ssDNA was incubated with an increasing amount of *Pha*SSB and the resulting complexes were analyzed using agarose gel electrophoresis. As shown in Fig. 4B when the SSB-ssM13 DNA complexes are formed by direct fixing at a low concentration of NaCl (2 mM), the electrophoresis pattern indicates highly cooperative binding of SSB. In particular, there are streaking in lanes 32,

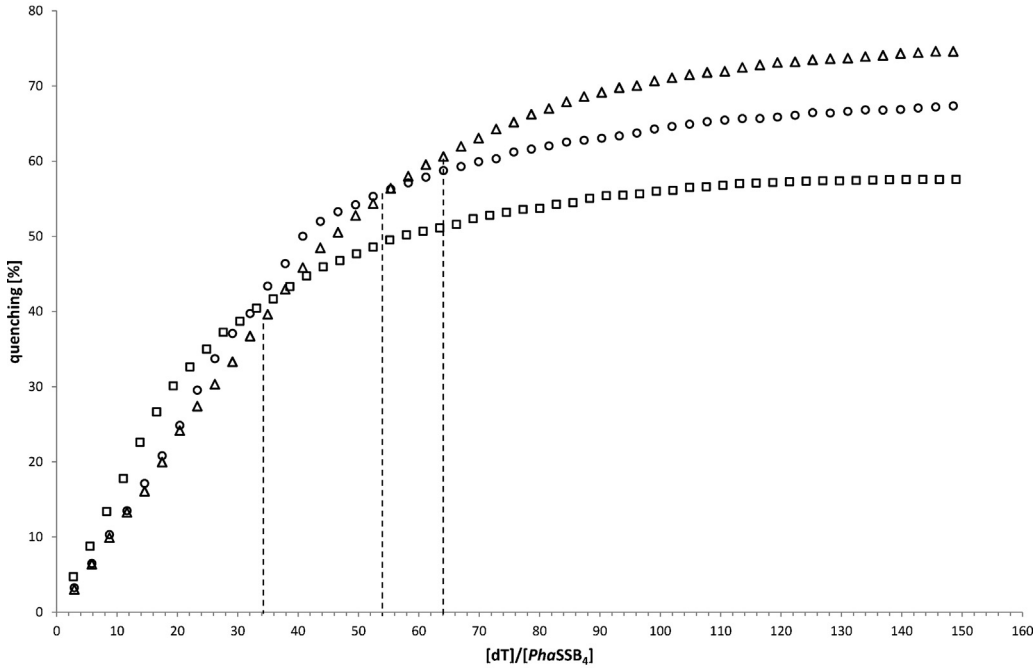

Fig. 5. Inverse fluorescence titration of *Pha*SSB with poly$(dT)_{250}$. A 1 nmol sample of SSB protein was titrated with $(dT)_{250}$ at 2 mM (□), 100 mM (○) and 300 mM (Δ) NaCl binding buffer.

Table 2
Percentage amino acid content of the *Eco*SSB, *Tma*SSB, *Tte*SSB3 and *Pha*SSB proteins.

SSB	Ala	Ile	Leu	Val	Met	Gly	Pro	Lys	Arg	Asp	Glu	Gln	Asn	Ser	Thr	His	Trp	Phe	Tyr	Cys
*Pha*SSB	5.7	2.9	2.5	4.9	2.5	15.6	7	4.1	2.5	4.5	3.3	18.9	6.6	6.1	4.5	0.4	0.8	2.9	4.1	0.4
*Eco*SSB	7.3	2.8	4.5	7.3	3.4	16.3	6.7	3.4	5.6	4.5	5.6	10.1	4.5	5.6	5.0	0.6	2.2	2.2	2.2	0
*Tte*SSB3	4.0	5.3	7.3	8.7	2.0	6.0	6.0	5.3	6.0	10.7	8.0	1.3	4.0	6.7	8.0	0.7	2.0	6.0	1.3	0
*Tma*SSB	5.0	4.3	5.7	9.2	2.8	4.3	7.1	3.5	10.6	6.4	12.8	0.7	2.1	5.0	10.6	0	0.7	7.8	1.4	0

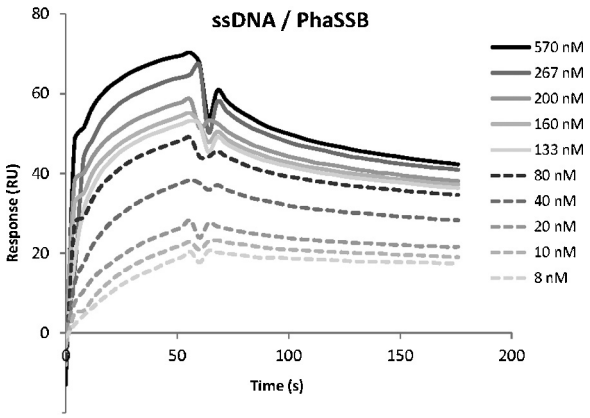

Fig. 6. Interaction analysis of *Pha*SSB with ssDNA. Different concentrations of the protein were injected with a flow rate of 30 μl/min on a streptavidin chip coated with ssDNA 60-mer. A flow cell with streptavidin was used as a reference. After each injection, the chip was regenerated with 0.01% SDS. The colors of the sensograms represent the concentrations of *Pha*SSB injected. The data were analyzed in accordance with the Langmuir model and using BiaEval 3.0 software.

60, 70, 80 and 100 pmol of the gel and a discrete band is formed that migrates slower than the rest of the DNA population, indicating DNA molecules that are highly saturated with SSB. This would be consistent with how *E. coli* SSB interacts with M13 ssDNA under low-salt conditions (Lohman et al., 1986).

In case of 300 mM of NaCl the banding patterns are different and show only a single somewhat diffuse band at each SSB to DNA ratio (Fig. 4C) indicating low co-operativity. This result is similar as for *E. coli* SSB under higher salt conditions, with tighter bands, which indicate less cooperative binding under those conditions (Lohman et al., 1986).

To further explore the binding properties of *Pha*SSB, we used fluorescence spectroscopy. All the bacterium SSBs studied to date have shown a dramatic decrease of tryptophan fluorescence when binding to ssDNA. With an excitation wavelength of 295 nm, the emission spectrum of the SSB proteins at 25 °C reached its maximum at 348 nm, which is consistent with tryptophan fluorescence. On the addition of a saturating quantity of $(dT)_{250}$, the intrinsic fluorescence at 348 nm was quenched by 58, 67 and 74% in 2, 100 and 300 mM NaCl containing buffers. The estimated binding site in the presence of 2, 100 and 300 mM NaCl was determined as 34 ± 2, 55 ± 2 and 64 ± 2, respectively (Fig. 5). These results show a considerable binding mode transition when changing the salt concentrations from low to high, much like that observed for *Eco*SSB.

The binding properties of *Pha*SSB were also investigated with SPR technology (Fig. 6). Using a streptavidin chip coated with a single-stranded DNA fragment of 60 nt, the kinetic parameters were estimated. The affinity of *Pha*SSB to ssDNA, as reflected in a K_d value of pico-molar range (Table 1), is typical of SSB proteins such as *Eco*SSB or *Mtu*SSB, determined using the same ssDNA 60-mer (Reddy et al. 2001; Ehn et al. 2001). In addition, a control measurement of binding recombinant *Eco*SSB to ssDNA was performed as part of the study (Table 1). The only difference in the binding mode of *Pha*SSB to ssDNA is a faster association phase, when compared to *Eco*SSB, although this is compensated for by a faster dissociation rate.

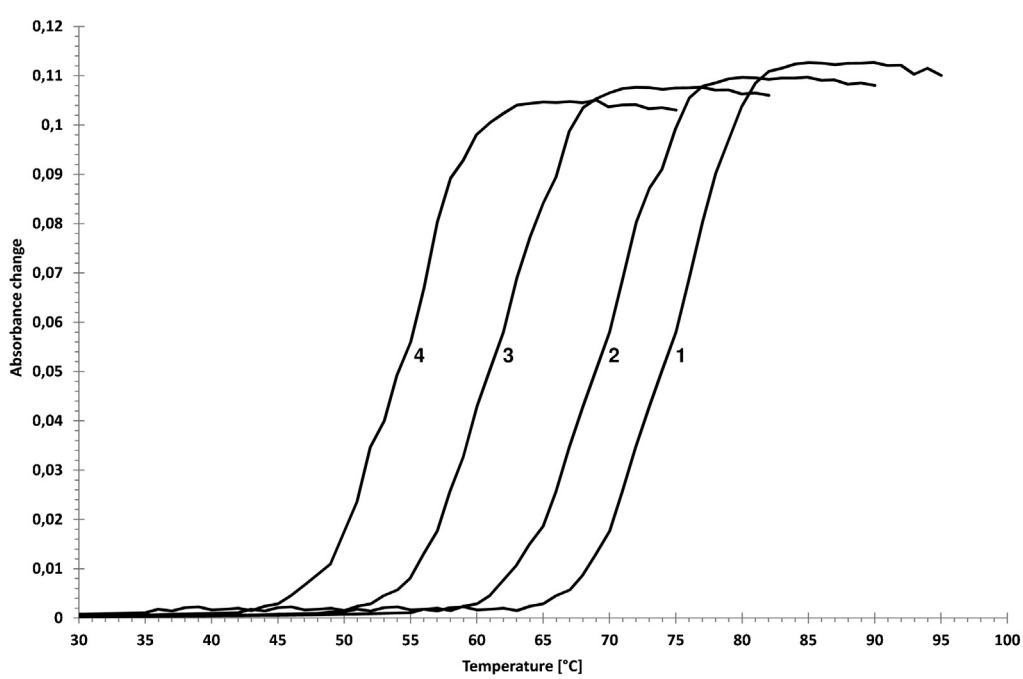

Fig. 7. Melting profiles of dsDNA and its complexes with SSB proteins. A 1 mol sample of duplex DNA (44 bp) was incubated alone (1) and with 5 pmol of *Tma*SSB (2), *Eco*SSB (3) or *Pha*SSB (4), in a standard buffer containing 0.1 NaCl. Absorbance changes were measured at 260 nm.

3.5. Melting point destabilization of dsDNA

As a thermodynamic consequence of SSB proteins binding specifically to ssDNA and not to dsDNA, a destabilization of DNA double strands in the presence of SSBs must be expected. The results of the destabilization of duplex DNA (44 bp) by *Pha*SSB, *Eco*SSB and *Tma*SSB are shown in Fig. 7. The melting temperature of duplex DNA in 0.1 M NaCl is decreased from 75 to 55 °C by *Pha*SSB. In comparison, under the same conditions, the melting temperature of the dsDNA in question is decreased from 75 to 62 °C and from 75 to 70 °C by *Eco*SSB and *Tma*SSB, respectively. The experiments were repeated three times with the same results.

3.6. Thermostability

When analyzed by differential scanning microcalorimetry (DSC), the thermal unfolding of *Pha*SSB, *Eco*SSB and *Tma*SSB was found to be an irreversible process, as seen in the rescan thermogram (Fig. 8). As expected, the *Pha*SSB had the lowest thermostability, with a melting temperature (T_m) of 63.0 °C, whereas *Eco*SSB and *Tma*SSB had a T_m of 69.0 and 109.3 °C, respectively. The thermograms of these SSB proteins showed none of the characteristic signs of heavily aggregated proteins after heat denaturation. Although *Pha*SSB protein comes from psychrophilic microorganism, it has a relatively high thermostability.

4. Discussion

In this article, we have described the purification and characterization of single-strand DNA-binding protein (*Pha*SSB) from the facultative psychrophilic bacteria *P. haloplanktis*. The *Pha*SSB forms a tetramer in solution, as demonstrated by the analytical ultracentrifugation and chemical cross-linking experiments. The results of the sequence analysis verified that a ssDNA binding domain (the first 106 amino acid residues) in one monomer of *Pha*SSB protein possesses a canonical oligonucleotide binding fold (OB-fold) very similar to that observed in the structure of *E. coli* SSB. The *Pha*SSB sequence is far more similar to the sequence of *Eco*SSB than to that of the *Tte*SSB3 and *Tma*SSB proteins isolated from thermophilic bacteria (Dąbrowski et al. 2002b; Olszewski et al. 2008, 2010). A 75% identity and 90% homology of the *Pha*SSB OB-fold domain to *Eco*SSB was somewhat surprising, given that they come from taxonomical distant organisms which live in different environments. Furthermore, *Pha*SSB possesses the longest C-terminal domain (from 107 to 244 amino acid residues) among the SSBs with one OB-fold domain per monomer described to date. The C-terminal domain contains a high number of negatively charged residues, which are required for the interaction of SSBs with various proteins in *E. coli* and other eubacteria but are not essential for DNA binding. There are also five conserved DDIPF terminal amino acid residues, which are common in all the known bacterial SSB proteins.

In *E. coli*, the SSB base-stacking residues are Trp-40, Trp-54, Phe-60 and Trp-88, and in contrast to *Tma*SSB or *Tte*SSB3, the location of these residues is precisely preserved with the one amino acid residue shift in *Pha*SSB (Tyr-39, Trp-53, Phe-59, Trp-87). Tyrosine is present at position 39 in *Pha*SSB instead of tryptophan; the former is also aromatic amino acid residue and can play an role analogical to that of Trp40 in *Eco*SSB, participating in ssDNA binding. Highly conserved His-55, Gln-76 and Gln-110, important for the homotetramerization of *Eco*SSB, are also present in *Pha*SSB protein.

The *Pha*SSB had the lowest thermostability, with a melting temperature (T_m) of 63.0 °C, in comparison to *Eco*SSB and *Tma*SSB, with T_m of 68.0 and 109.3 °C, respectively. This relatively high thermostability for *Pha*SSB was surprising in the context of the psychrophile *P. haloplanctis* and in comparison with the SSB from mesophilic *E. coli*. It is generally accepted that, overall, cold-adapted proteins are more flexible than their mesophilic counterparts, with a reduced number of weak interactions. This flexibility often coincides with reduced thermal stability of the psychrophilic protein.

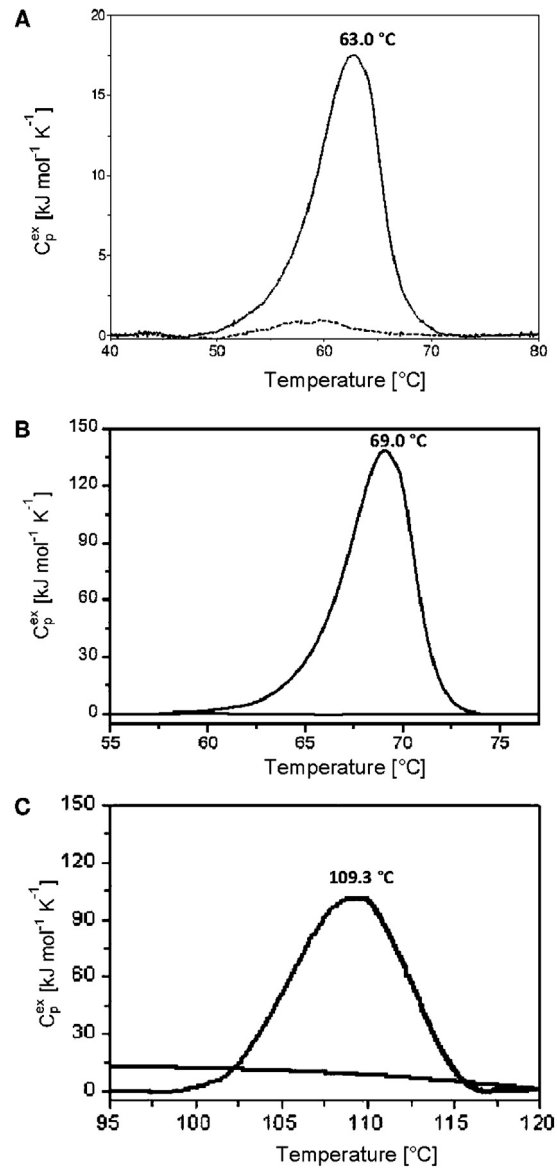

Fig. 8. DSC termograms of SSB proteins. Samples containing 1.5 mg/ml *Pha*SSB (panel A) or *Eco*SSB (panel B) or *Tma*SSB (panel C) were analyzed in 50 mM of potassium phosphate buffer pH 7.5 and 150 mM NaCl. The melting temperatures are shown.

In studies of other SSBs, it has often been shown that the size of the binding site depends on the salt concentration. For example, at least two distinctly different DNA-binding modes have been described for *Eco*SSB, (Lohman and Overman 1985). In high salt concentrations, 65 nt bind per *Eco*SSB tetramer with a fluorescence quench of almost 90%, whereas in low salt concentrations, 35 nt are sufficient to saturate the protein and quench its fluorescence by only 53%. Our current study has demonstrated that the binding site size of *Pha*SSB is much the same as that observed for *Eco*SSB, with two distinctly different DNA-binding modes dependent on salt concentrations.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

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