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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.