

## Identification of the *csp* gene and molecular modelling of the CspA-like protein from Antarctic soil-dwelling psychrotrophic bacterium *Psychrobacter* sp. B6

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We cloned and sequenced the *cspA*-like gene from a psychrotrophic Antarctic soil-dwelling bacterial strain *Psychrobacter* sp. B6. The gene is 213 bp long and shows 99% and 98% sequence identity with the *Psychrobacter cryohalolentis* K5 gene encoding a cold-shock DNA-binding domain protein and the *Psychrobacter arcticus* transcriptional regulator-CspA gene, respectively. The protein encoded by the *Psychrobacter* sp. B6 *cspA*-like gene shows 100% identity with the two proteins mentioned above, and also 61% sequence identity with CspB from *Bacillus subtilis* and Csp from *Bacillus caldolyticus*, and 56% — with *Escherichia coli* CspA protein. A three-dimensional model of the CspA-like protein from *Psychrobacter* sp. B6 was generated based on three known structures of cold shock proteins: the crystal structure of the major cold shock protein from *Escherichia coli* (CspA), the NMR structure of the latter protein, and the NMR structure of Csp from *Thermotoga maritima*. The deduced structure of the CspA-like protein from *Psychrobacter* sp. B6 was found to be very similar to these known structures of Csp-like proteins.

**Keywords:** Cold-living bacterium, cold shock protein, CspA-like protein, RNA chaperone, nucleic acid binding

### INTRODUCTION

Throughout evolution live organisms have developed many adaptations to harsh and hostile environmental conditions and the changes in these conditions. A sudden temperature downshift is one of principal environmental stresses encountered by bacteria, in particular those inhabiting the permanently cold areas of the Earth. It results in a significant decrease in cell membrane fluidity and disturbs virtually all intracellular processes, including transcription and translation (Russell *et al.*, 1995; Jones & Inouye, 1996). To survive under such adverse conditions, microorganisms produce a number of cold-

induced proteins. A family of small proteins named cold shock proteins (Csp) is the most essential for cell survival.

The first cold shock protein CspA from *Escherichia coli* was identified in 1987 (Jones *et al.*, 1987). From that time, the cold shock response has been extensively investigated. In contrast to the heat shock response it is, however, still not so well understood.

The cellular cold shock response seems to be universal (Jones & Inouye, 1994) as it occurs in almost every microbial cell after a rapid drop of temperature and always brings about synthesis of specific proteins — the cold shock proteins. CspA

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**Abbreviations:** Csp, cold shock protein; CSD, cold shock domain; NMR, nuclear magnetic resonant; OB-fold protein, oligonucleotide binding protein; RNP, single stranded nucleic acids binding motif; ssDNA, single-stranded DNA.

have been found in all types of bacteria, i.e. in thermophiles, mesophiles and psychrophiles (Mueller *et al.*, 2000; Ermolenco & Makhatadze, 2002; Phadtare *et al.*, 2003). Several homologous copies of *csp* genes have been identified in the majority of known bacterial genomes. Nine *csp* homologues, *cspA–cspI*, were found in *E. coli* (Yamanaka *et al.*, 1998), five of these genes are expressed in response to a sudden drop in temperature (Nakashima *et al.*, 1996; Wang *et al.*, 1999; Uppal *et al.*, 2008). Three *csp* genes, *cspB*, *C* and *D* were found in *B. subtilis* (Schindler *et al.*, 1999) and two cold shock genes, *cspA* and *cspG*, both cold-induced, were identified in a deep-sea psychrophilic bacterium *S. violacea* (Fujii *et al.*, 1999). Four genes encoding a small cold-induced Csp from the Antarctic *Streptomyces* sp. AA 8321 have also been cloned recently (Kim *et al.*, 2007).

Csps are believed to act as regulators of transcription and translation (Gualerzi *et al.*, 2003). They also act as molecular chaperones because they non-specifically bind single-stranded nucleic acids and destabilize their secondary structures at low temperature (Jiang *et al.*, 1997; Lopez & Makhatadze, 2000; Bae *et al.*, 2000; Phadtare *et al.*, 2002; Zeeb & Balbach, 2003; Zeeb *et al.*, 2006). In general, Csps are involved in establishing a “new” cell balance, which in turn facilitates survival at low temperature.

It is postulated that the cold shock proteins also enhance microbial tolerance to freezing due to cryoprotectant properties (Willmsky *et al.*, 1992). It was found that viability of cells of some bacterial strains increased when they had been subjected to a cold-shock prior to freezing. It implies that the cold-shock invokes cryotolerance (Kim & Dunn, 1997; Jeffreys *et al.*, 1998; Kim *et al.*, 1998).

Studies on cold shock proteins have focused on those produced by meso- and thermophiles. Three dimensional structures have been determined for four cold shock proteins from mesophilic, thermophilic and hyperthermophilic bacteria such as: *Escherichia coli* Ec-CspA (Newkirk *et al.*, 1994; Schindelin *et al.*, 1994; Feng *et al.*, 1998), *Bacillus subtilis* Bs-CspB (Schuchel *et al.*, 1993; Schindelin *et al.*, 1993), *Bacillus caldolyticus* Bc-Csp (Mueller *et al.*, 2000) and *Thermotoga maritima* Tm-Csp (Kremer *et al.*, 2001). All of these small cold shock proteins have a very similar conformation and contain five anti-parallel  $\beta$ -strands folded into a  $\beta$ -barrel structure. This particular molecule fold is characteristic for the OB-fold family of proteins (Ermolenko & Makhatadze, 2002; Horn *et al.*, 2007). Interestingly, eukaryotic cold shock domains (CSDs), located in Y-box proteins, are involved in nucleic acid binding and show relatively high sequence and structure similarity to prokaryotic Csps (Kloks *et al.*, 2002; 2004).

The three-dimensional structure of a psychrophilic or psychrotrophic Csp has not yet been

determined. We have modelled the 3D structure of the CspA-like protein from the psychrotrophic bacterium *Psychrobacter* sp. B6 on the basis of the sequence of its gene. This is the first model describing a Csp molecule from a cold-adapted bacterium.

## MATERIALS AND METHODS

**Bacterial strain and growth conditions.** The psychrotrophic bacterial strain B6 was isolated from samples of Antarctic soil derived from the coast of Admiralty Bay, King George Island (close to the Henryk Arctowski Polish Antarctic Station). The strain B6, a Gram-negative coccobacillus, was identified as *Psychrobacter* sp. based on partial sequence of its 16S rRNA gene (deposited in GenBank/EMBL, accession number EF028072).

Shaken cultures of this strain were grown at 20°C and 130 r.p.m. in a liquid nutrient medium (bacto beef extract, 0.3%; bacto peptone, 0.5%; yeast extract, 0.25%; NaCl, 0.5%; glucose, 0.1%) until the OD<sub>540</sub> reached 0.4–0.6.

**DNA isolation.** Cells from 5-ml cultures were harvested by centrifugation at 12000×g at 4°C for 20 min. The pellet was suspended in 200  $\mu$ l TE buffer (10 mM Tris/HCl, pH 8.0, 1 mM EDTA) and treated with 10 mg ml<sup>-1</sup> lysozyme at 37°C for 12 h. Two drops of glass beads (Invitrogen) suspended in TE buffer were added and the cells were mechanically disrupted using a Braun MKS grinder for 1 min, at maximal speed. Then the cells were incubated with DNAzol Reagent (Invitrogen) in accordance with the manufacturer's instruction. The glass beads and unbroken cells were removed by centrifugation at 12000×g at 4°C for 10 min. The lysate was extracted three times with phenol/chloroform/isoamyl alcohol (25:24:1, by vol.) and then DNA was precipitated with 95% ethanol and dissolved in nuclease-free water.

**PCR amplification.** The PCR primers Psch 8 forward: 5'-AATCGGTGATGACACACGTC-3' and Psch 8 reverse: 5'-TACTGTCATTAAGCGCGCTG-3' were designed based on flanking regions of two major Csp gene sequences from *Psychrobacter cryohalolentis* K5 and *Psychrobacter arcticus* 273-4 (NCBI Genome Database, acc. no. NC\_007969 and NC\_007204, respectively). The amplified PCR product was 330 bp in length. PCR amplification was performed in a T-Personal thermocycler (Biometra) using 1 U of *Taq* polymerase (Invitrogen). PCR conditions were as follows: denaturation at 94°C for 3 min, followed by amplification for 35 cycles at 94°C for 1 min, 52°C at 1 min, and 72°C for 2 min, followed by a final extension at 72°C for 8 min. The amplified DNA fragment was purified by electrophoresis in 1.5% agarose gel (in TAE buffer). The gels were stained with ethid-

ium bromide (1  $\mu\text{g ml}^{-1}$ ) and analyzed in UV light (Fc8800, Alphainnotech).

**Cloning of the *csp*-like gene from *Psychrobacter* sp. B6.** Cloning of the PCR product harboring the *csp*-like gene was performed using the pGEM T-easy system (Promega) and DNA mixtures were transformed into *E. coli* DH5 $\alpha$ . The clones were selected by the X-gal/IPTG screening method (Sambrook & Russell, 2001). Plasmid DNA was prepared by alkaline lysis of culture using a Promega kit (Vizard Plus SV Minipreps) and five independent clones were sequenced at the DNA Sequencing and Oligonucleotide Synthesis Laboratory, Institute of Biochemistry and Biophysics, Polish Academy of Sciences (Warszawa, Poland). The nucleotide sequence of the CspA-like protein gene from *Psychrobacter* sp. B6 was deposited in the GenBank/EMBL nucleotide databases (accession number EF094463).

**Sequence data analysis and molecular modelling.** GenBank was scanned for related sequences by the BLAST algorithm. Nucleotide and amino-acid sequences were aligned by using the computer program Vector.

Protein structure prediction was carried out via the GeneSilico MetaServer (<http://genesilico.pl/meta/>, Kurowski & Bujnicki, 2003), which is a convenient gateway to a number of publicly available online services for secondary structure prediction.

The alignment between the sequences of the CspA-like protein from *Psychrobacter* sp. B6 and its counterparts from the database was the first step of homology modelling. A set of alternative models was generated using MODELLER (Sali & Blundell, 1993) and SWISS-MODEL (Peitsch, 1996), and the models were scored using VERIFY3D (Luthy *et al.*, 1992) via the COLORADO3D server (Sasin & Bujnicki, 2004). The final model was obtained using the 'Frankenstein's monster' approach (Kosinski *et al.*, 2003) which comprises cycles of realignment in poorly scored regions and merging the best scoring fragments. The sequence-structure fit in the final model was also evaluated using VERIFY3D (Luthy *et*

*al.*, 1992), while the stereochemical parameters were assessed with PROCHECK (Laskowski *et al.*, 1993).

Modelling of the *Psychrobacter* sp. B6 CspA-like protein was performed using templates selected based on the results of the GeneSilico MetaServer. The templates used for the modelling were PDB ID: 1mjc — the crystal structure of the major cold shock protein from *E. coli* (CspA), (Schindelin *et al.*, 1994), PDB ID: 3mef — the NMR structure of the same protein solved by Feng *et al.* (1998), and PDB ID: 1g6p — the NMR structure of Csp from *Thermotoga maritima* (Kremer *et al.*, 2001). However, since the superposition of all the aforementioned structures gave a highly consistent topology, as the final template only the X-ray structure was used.

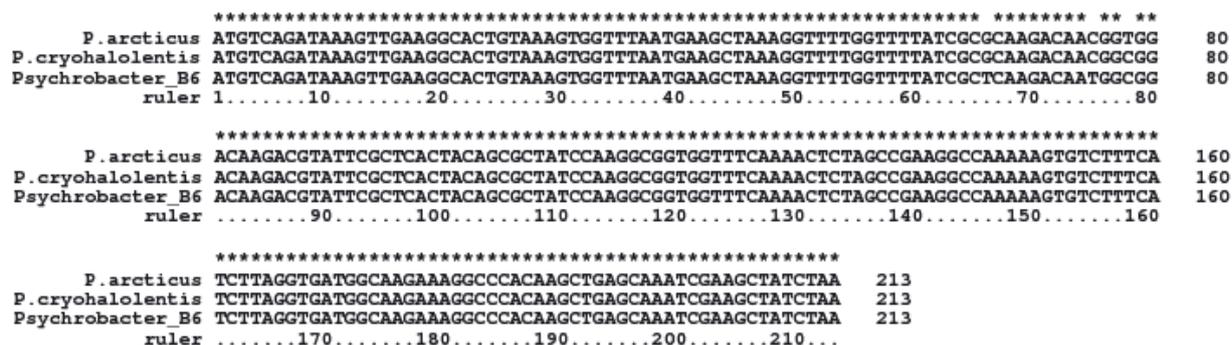
## RESULTS

### Cloning and sequencing of CspA-like gene from *Psychrobacter* sp. B6

The predicted *cspA*-like ORF from *Psychrobacter* sp. B6 consists of 213 bp and shows 99% sequence identity with the gene of a DNA-binding domain protein from *Psychrobacter cryohalolentis* K5 and 98% with the gene of the transcriptional regulator-CspA from *Psychrobacter arcticus* 273-4 (Fig. 1). The predicted amino-acid sequence of the protein encoded by *cspA*-like gene is identical (Fig. 2) with that of the cold-shock DNA-binding domain protein from *P. cryohalolentis* K5 and the transcriptional regulator-CspA from *P. arcticus* 273-4. Besides, these proteins display 56% identity with *E. coli* major cold shock protein CspA, and 61% with *B. subtilis* CspB and *B. caldolyticus* Csp (Fig. 3).

### Molecular modelling of CspA-like protein

Modelling of the *Psychrobacter* sp. B6 CspA-like protein was based on known structures of major cold shock proteins from *E. coli* (CspA), (Schin-



**Figure 1.** Sequence alignment of genes encoding cold shock proteins from *Psychrobacter* genus. *Psychrobacter arcticus* 273-4, transcriptional regulator CspA (NC\_007204); *Psychrobacter cryohalolentis* K5, cold-shock DNA-binding domain protein (NC\_007969); *Psychrobacter* sp. B6 CspA-like (EF094463).

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AATCGGTGATGACACACGCTCTGCCAACAGTTAAAGAGTTAGGAAAATTTATGTCAGTAAA
- - - - - M S D K
GTTGAGGCACTGTAAGTGGTTTAAATGAGCTAAGGTTTGGTTTATCGCTCAAGAC
V E G T V K W F N E A K G F G F I A Q D
AATGGGGACARGAGCTATTTCGCTACTACAGCGCTATCCAAAGCGGTGGTTTCAAAC
N G G Q D V F A H Y S A I Q G G G F K T
CTAGCGAAGGCCAAAAGTGTCTTTCATCTTAGGTGATGGCAAGAAAGGCCCAAGCT
L A E G Q K V S F I L G D G K K G P Q A
GAGCAATCGAAGCTATCTAATCTATTGACATTGAGCGAGCTAATTTTTCAGCGCGCT
E Q I E A I * - - - - -
TAATGACAGTA
- - -

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Figure 2. Nucleotide (313 bp) and predicted amino-acid sequences of *Psychrobacter* sp. B6 *cspA* region.

The *cspA*-like coding sequence is shown in bold letters; an asterisk indicates the stop codon.

delin *et al.*, 1994; Feng *et al.*, 1998) and *T. maritima* (Kremer *et al.*, 2001). The homology model of the CspA-like protein shows that, like other Csp, the Csp molecule from *Psychrobacter* sp. contains five anti-parallel  $\beta$ -strands that fold into a  $\beta$ -barrel built of two  $\beta$ -sheets. One of these  $\beta$ -sheets, consisting of strands  $\beta_1$ - $\beta_2$ - $\beta_3$ , contains two nucleic acid binding motifs on its surface (Fig. 4). The first one, RNP1 – KGFGFI is the same as in the majority of known Csp proteins. The RNP2 motif – VFA/VHY/F is characterized by two changes in amino-acid composition in comparison to the majority of known Csp protein sequences (Fig. 3). Seven aromatic amino-acid residues – W11, F12, F18, F20, F31, Y34 and F42, which are essential for single-stranded nucleic acid binding (Fig. 4) and two lysine residues, K10 and K16, are located on the surface. Thus the 3D structure of the Csp-like protein from *Psychrobacter* sp. B6 is similar to that of other known Csp molecules. Therefore we assume that Csp B6 plays the role of a molecular chaperone.

## DISCUSSION

CspA-like proteins have been found in cells of many bacterial species but studies on their function and cold shock response focused on mesophilic

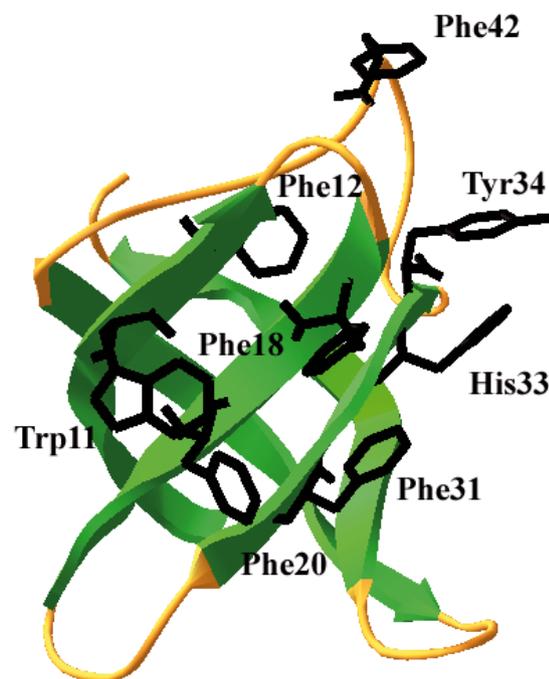


Figure 4. Three-dimensional molecular structure of the CspA-like protein from *Psychrobacter* sp. B6.

Aromatic residues essential for single stranded nucleic acid binding are highlighted in black.

and thermophilic microorganisms. These proteins have also been detected in psychrophilic and psychrotrophic bacteria, such as *Pseudomonas fragi* (Hebraud *et al.*, 1994), *Arthrobacter globiformis* (Berger *et al.*, 1996), *Shewanella violacea* (Fujii *et al.*, 1999) and strains isolated from polar regions like: *Colwellia* sp. NJ341 (Wang *et al.*, 2006) and *Streptomyces* sp. AA8321 (Kim *et al.*, 2007). However, 3D structures of those Csp, have not been determined yet.

Sequences of Csp, are highly conserved regardless of their source. Because even subtle differences in amino-acid composition and in 3D structure among Csp, can lead to differences in their physiological function and interactions with other biomolecules, elucidation of these aspects is necessary.

	RNP1	RNP2	
	* ****: ***:*	: - ***:*	***: **:* ** * * * : * : * * * * : :
<i>B. caldolyticus_Csp</i>	---MQRGKVKWFNNEKGYGFIEVEGGSDVVFVHF	TAIQGEGFKTLEEGQVWSEFIVQGNRGPQAANVVKL	66
<i>B. subtilis_CspB</i>	---MLEGKVKWFNNEKGYGFIEVEGGSDVVFVHF	BAIQGEGFKTLEEGQVWSEFIVQGNRGPQAANVTKBA	67
<i>T. maritima_Csp</i>	---MRGKVKWFNNEKGYGFITKDEGGSDVVFVHF	BAIQGEGFKTLEEGQVWSEFIVQGNRGPQAANVTKBA	66
<i>E. coli_CspA</i>	MSGKMTGIVKWFNNEKGYGFITPDGSGKDVVFVHF	BAIQNDGYSKLDGQKVSFTIESGAKGPAAGNVTSL	70
<i>P. arcticus_CspA</i>	MSDKVEGTVKWFNNEKGYGFIAQDNGGQDVFVHF	BAIQGGGFKTLAEGQKVSFILGDGKRGPAEQIEAI	70
<i>P. cryohalolentis_Csp</i>	MSDKVEGTVKWFNNEKGYGFIAQDNGGQDVFVHF	BAIQGGGFKTLAEGQKVSFILGDGKRGPAEQIEAI	70
<i>Psychrobacter_sp.B6_CspA</i>	MSDKVEGTVKWFNNEKGYGFIAQDNGGQDVFVHF	BAIQGGGFKTLAEGQKVSFILGDGKRGPAEQIEAI	70
ruler	1.....10.....20.....30.....	40.....50.....60.....70.....	

Figure 3. Sequence alignment (CLUSTAL X algorithm) of the CspA-like protein from *Psychrobacter* sp. B6 with Csp sequences from other bacteria.

The analyzed sequences were obtained from GenBank database: *Bacillus caldolyticus* Bc-Csp (1C9O\_A), *Bacillus subtilis* CspB (NP\_388791), *Thermotoga maritima* Csp (O54310), *Escherichia coli* CspA (ACC76580), *Psychrobacter arcticus* (YP\_263828), *Psychrobacter cryohalolentis* Csp (YP\_579793). Boxes indicate RNA/ssDNA binding motifs.

Cloning and sequencing of the *cspA*-like gene from Antarctic *Psychrobacter* sp. B6 allowed the modelling of the three-dimensional structure of the protein encoded by this gene. This model provides evidence that the CspA-like protein from *Psychrobacter* sp. B6 shows high structural similarity to known three-dimensional structures of CspA. The CspA-like molecule consists of two  $\beta$ -sheets (I: strands  $\beta_1$ - $\beta_3$ ; II: strands  $\beta_4$  and  $\beta_5$ ) folded into a five-stranded  $\beta$ -barrel structure. Like in the other CspA with known 3D structures, the single-stranded nucleic acid binding motifs, RNP1 and RNP2, are located on the surface of the first  $\beta$ -sheet. Seven aromatic residues (W11, F12, F18, F20, F31, H33 and Y34) are also exposed on the surface of this  $\beta$ -sheet while residue F42 is located in the coil region (Fig. 4). Also two lysine residues (K10, K16) with positively charged side chains are located in the same region of the molecule. Aromatic residues, especially those located in RNP1 and RNP2 motifs, are essential for the tight binding of single-stranded nucleic acids (Schröder *et al.*, 1995; Schindler *et al.*, 1998). The other aromatic and non-aromatic side chains are believed to take part in intercalation of the RNA or DNA bases and therefore stabilization of the RNA/DNA-Csp complex.

KGFGFI is the most common sequence of CspA' RNP1 motifs, including the *Psychrobacter* sp. B6 RNP1 motif. Two phenylalanine aromatic residues of RNP1 are essential for tight binding of RNA/ssDNA. Substitution at these positions in RNP1 of *B. subtilis* CspB with non-aromatic residues reduced the binding affinity (Zeeb & Balbach, 2003). Interestingly, there is one difference in RNP1 motif composition of thermophilic bacteria such as *B. caldolyticus* (Mueller *et al.*, 2000) and *T. maritima* (Kremer *et al.*, 2001). They contain Tyr instead of Phe (KGYGFI). Analysis of an *Bs*-Csp variant with Tyr15 in place of Phe15 revealed a two-fold higher binding affinity as compared to the wild-type CspB (Zeeb & Balbach, 2003).

The amino-acid composition of the RNP2 nucleic acid binding motif in *Psychrobacter* sp. B6 CspA-like protein is different from that of the majority of known motifs in mesophilic and thermophilic CspA. For example, the RNP2 motifs of *E. coli* (Yamanaka *et al.*, 1998), *B. subtilis* (Schröder *et al.*, 1995) and *B. caldolyticus* (Mueller *et al.*, 2000) have the sequence VFVHF while in our protein this sequence is VFAHY. It is worth noting that the VFAHY sequence of the RNP2 motif is conserved in the other known CspA from species of *Psychrobacter* (*P. cryohalolentis* and *P. arcticus*) and *Streptomyces* genera, including an Antarctic strain — *Streptomyces* sp. AA8321 (Kim *et al.*, 2007). For comparison, the RNP2 motif in Csp of the polar psychrophilic bacterium *Colwellia* sp. NJ341 is VVFHY (Wang *et al.*, 2006). Thus, the occurrence of a tyrosine residue as the last amino acid of

this motif may suggest that this feature is common of the CspA from cold adapted bacteria and distinguishes them from their mesophilic counterparts.

The tyrosine residue is replaced by phenylalanine in the RNP2 motif of *B. subtilis* CspB. Mutation studies have revealed that the highly conserved phenylalanine residues in RNP1 and RNP2 are important for nucleic acid binding and stabilization of this complex (Zeeb & Balbach, 2003, Zeeb *et al.*, 2006). A Phe→Ala substitution in CspB nucleic acid binding motifs reduced the protein binding affinity. Conversely, replacement of Phe30 with Tyr in RNP1 motif of *B. subtilis* CspB caused a two-fold increase in ssDNA binding affinity in comparison to the wild-type CspB (Zeeb & Balbach, 2003). In this context, it is possible that the occurrence of Tyr30 (instead of Phe) in RNP2 motifs of CspA-like proteins from cold adapted bacteria can increase their affinity for single-stranded nucleic acids.

In conclusion, the 3D structure of the CspA-like protein from *Psychrobacter* sp. B6 indicates that it plays a role of a molecular RNA/ssDNA chaperone but this hypothesis needs to be verified experimentally since comprehension of the cold shock response mechanism and cold adaptation of psychrophilic and psychrotrophic microorganisms requires clear defining functions of CspA proteins and regulation of their expression.

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