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CspB of an arctic bacterium, Polaribacter igrans, KOPRI 22228, confers extraordinary freeze-tolerance

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Abstract

Freezing temperatures are a major challenge for life at the poles. Decreased membrane fluidity, uninvited secondary structure formation in nucleic acids, and protein cold-denaturation all occur at cold temperatures. Organisms adapted to polar regions possess distinct mechanisms that enable them to survive in extremely cold environments. Among the cold-induced proteins, cold shock protein (Csp) family proteins are the most prominent. A gene coding for a Csp-family protein, cspB, was cloned from an arctic bacterium, Polaribacter igrans KOPRI 22228, and overexpression of cspB greatly increased the freeze-survival rates of Escherichia coli hosts, to a greater level than any previously reported Csp. It also suppressed the cold-sensitivity of an E. coli csp-quadruple deletion strain, BX04. Sequence analysis showed that this protein consists of a unique domain at its N-terminal end and a well conserved cold shock domain at its C-terminal end. The most common mechanism of Csp function in cold adaption is melting of the secondary structures in RNA and DNA molecules, thus facilitating transcription and translation at low temperatures. P. igrans CspB bound to oligo(dT)-cellulose resins, suggesting single-stranded nucleic acid-binding activity. The unprecedented level of freeze-tolerance conferred by P. igrans CspB suggests a crucial role for this protein in survival in polar environments.

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Introduction

Living organisms encounter several serious challenges when they are exposed to cold environments, including reduced enzyme activity, decreased membrane fluidity, reduced transport of nutrients and waste products, reduced protein movement, decreased rates of transcription and translation, slow DNA replication, retarded protein folding, cold-denaturation of proteins, and intracellular ice formation.1-3 Although...
freezing temperatures are a major environmental threat at the North and South Poles, relatively high numbers of diverse microorganisms thrive in the polar regions. In particular, α-, β-, and γ-proteobacteria and the Cytophaga-Flavobacterium-Bacteroides phyla are the most commonly found bacteria, and eukaryotes, such as yeasts and microalgae, are frequently found in polar environments. Organisms living in the polar regions must have the ability to adapt to extremely cold temperatures. To avoid the transition from a fluid- to gel-phase cell membrane, the proportion of unsaturated and methyl-branched fatty acids in lipid membranes is increased. The formation of stable secondary structures in nucleic acids at low temperatures inhibits replication, transcription, and translation. Thus, nucleic acid-binding proteins, such as Csp-family proteins and RNA helicases, are induced during temperature downshifts and function as RNA chaperones, melting the stable secondary structures in RNA molecules, which facilitates translation and ribosome biogenesis. Several chaperones, such as GroEL and DnaK, are induced upon cold shock, possibly to cope with cold-denatured proteins. Protein folding, especially cis/trans isomerization of peptidyl prolyl bonds, occurs very slowly at low temperatures, and peptidyl prolyl isomases play an important role in cold adaptation by facilitating the folding of functionally significant proteins. Cryoprotectants, such as antifreeze proteins, trehalose, and exopolysaccharides, have also been implicated in protection of polar organisms, either by preventing ice crystal formation or by avoiding dehydration. Genomic sequencing results suggest some other characteristics of psychrophiles, like enhanced antioxidant capacity to cope with increased production of toxic reactive oxygen species.

Increasing numbers of Csp homologs are being reported from psychrophiles, mostly through genomic DNA sequencing projects, but only limited functional data are available. In an effort to elucidate the detailed mechanisms that allow polar organisms to survive at low temperatures, we studied the functional roles of Csp-family proteins in polar bacteria. A psychrophilic bacterium, Polaribacter irgensii KOPRI 22228, was isolated from Arctic Sea sediment. Two csp genes, cspaB and cspC, were previously cloned from this bacterium and exhibited considerable homology to canonical Escherichia coli cspa (CspA). Overexpression of either of these two genes conferred significant cold-resistance phenotypes to their recombinant hosts, and also complemented the cold-sensitivity of a quadruple csp deletion mutant BX04 (Δcspa, Δcspb, Δcspg, and Δcsep) strain, suggesting functional homology among those Csp-family proteins. In this study, we report another Csp-homologous protein from P. irgensii KOPRI 22228, CspbB, which contains an extra domain on its N-terminal region, in addition to the well conserved cold-shock domain (CSD) at its C-terminal region. This belongs to a subfamily of CSD-fold proteins, recently identified through metagenomic studies of psychrophilic bacteria, and no functional data is available for this kind of proteins at our best knowledge. Therefore, the function of this unique Csp protein from an arctic bacterium in conferring cold tolerance was analyzed in this study.

Materials and methods

Bacterial culture and cloning of the cspbB gene

P. irgensii KOPRI 22228 was isolated from Arctic Sea sediments near Danan Korean Arctic Station (Ny-Alesund, Norway), and cultured as previously reported. The cspbB gene (GenBank WP_004570868) was obtained by PCR amplification. The template DNA was extracted from P. irgensii KOPRI 22228 cells using G-spin™ bacteria genomic DNA extraction kit (Invitrogen Co., Korea), according to the protocol suggested by the manufacturer. The forward primer sequence was CspbB F1H, 5′-AGTAAGCTTATTGCGAAAAATCCGACGACCTT-3′, and the reverse primer was CspbB R1, 5′-CCGGATCCTTATATTTTGGTAACTTTAACTGCATTCATT-3′ (manufactured by Bioneer Co., Daegon, Korea). The PCR mixture consisted of 5 μl of 10 × PCR buffer (final concentrations: 50 mM KCl, 0.1% gelatin, 10 mM Tris–HCl, pH 9.0), 2.5 mM MgCl2, 0.2 mM of each dNTP, 200 nM of each primer, 1 μl of template DNA, and 2.5 units of Taq DNA polymerase (Takara, Japan) in the final 50 μl volume. The PCR was performed in a DNAEngine thermal cycler (Bio-Rad Laboratories Inc., USA) using a cycling condition that consisted of an initial denaturation at 95 °C for 5 min and then 30 cycles with denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min. A final extension was performed at 72 °C for 5 min. The PCR products that were 0.5 kb in size were double-digested with HindIII and BamHI, and cloned using pAED4, an E. coli expression vector, digested with the same enzymes. To avoid any mutations arising from error-prone Taq DNA polymerase reactions, several clones were picked for sequencing analysis. The resulting plasmids was named pAED-cspbB.

Expression of CspbB in E. coli

The recombinant plasmid for expression of CspbB, pAED-cspbB, was transformed into competent E. coli BL21(DE3) cells (Invitrogen Co., CA, USA) using the method described by Sambrook et al. For overproduction of CspbB, 1 ml of the overnight liquid culture was transferred to a flask containing 50 ml fresh LB medium containing 100 μg/ml ampicillin. When cell cultures had reached an OD600 of 0.4, IPTG was added to the final concentration of 0.1 mM. The cultures were incubated further at 37 °C for 2 h with vigorous shaking. Overexpression of the CspbB, was analyzed by 20% SDS-PAGE. The protein bands were visualized by Coomassie brilliant blue R250 staining.

Resistance to freezing and thawing

CspbB protein was overexpressed in E. coli as described above. As the experimental control, E. coli transformed with a pAED4 plasmid lacking the cspbB insert was used. One ml aliquot of liquid culture was placed at −20 °C for 2 h. The frozen cells were taken out of the freezer and put on ice for 1 h to be thawed. This process was performed in duplicates and repeated up to three cycles. Aliquots were taken at each
cycle of freeze-and-thaw, and colony-forming units (CFU) were counted after incubation on LB plates at 37 °C for 24 h. The data were collected from five independent experiments and shown as an average for each point.

**Purification of CspBp**

CspBp were overexpressed at 37 °C in E. coli BL21(DE3) as described above, except that the cultures were scaled-up to 1 L liquid LB media. Cells were harvested by centrifugation, and resuspended in 40 ml of 10 mM phosphate buffer, pH 6.5. Cells were lysed by sonication using a Bandelin Sonoplus HD2200 ultrasonic homogenizer (Berlin, Germany) as described for CspAp. The supernatant fraction was loaded on a Q-sepharose™ (Amersham Bioscience Co.) fast flow ion exchange column equilibrated with the same buffer. CspBp protein was eluted with a 0–0.5 M NaCl gradient. Concentrations of proteins were determined using Bio-Rad DC (detergent compatible) protein assay kit, and the purity of proteins was analyzed by 20% SDS-PAGE.

**Oligo(dT)-cellulose binding assays**

Purified CspBp protein was dialyzed against binding buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 50 mM KCl, and 7.4% glycerol). Fifty μl of oligo(dT)-cellulose type 7 beads (Amersham Bioscience Co.) were incubated with 50 μg of CspBp protein at 4 °C for 4 h. The resins and bound proteins were collected by brief centrifugation, and washed twice with the binding buffer. In parallel, 50 μg of bovine serum albumin (BSA) protein, instead of CspBp protein, was used as the experimental control. Co-precipitated proteins were analyzed by 20% SDS-PAGE and Coomassie brilliant blue R250 staining.

**Rescue of cold-sensitive E. coli BX04 strain**

E. coli quadruple csp deletion strain BX04 cells harboring pAED, pAED-cspAp, pAED-cspBp, or pAED-cspCp were grown in the liquid culture to an OD600 of 0.4. The cells were then streaked onto LB plates containing 0.1 mM IPTG, and incubated at temperatures ranging from 16 to 37 °C. After 15 h at 37 °C, 24 h at 25 °C, or 60 h at 16 °C, the growth of BX04 cells on the plates was observed.

**Results**

**Cloning and sequence analysis of the cspB gene from P. igrنسي KOPRI 22228**

P. igrنسي KOPRI 22228 was isolated from Arctic Sea sediments and grown as previously reported. This bacterium is a psychrophile, with an optimum growth temperature of 10 °C. In an effort to elucidate the roles played by CspPs in psychrophilic bacteria, DNA fragments from this bacterium, which contain a region encoding a conserved CSD, were cloned. A novel csp-homologous gene 0.45 kb in size was obtained and named cspBp.

A BLAST nucleotide homology search of cspBp was performed: cspBp exhibited low homology to the canonical cspAp (15.8%) and E. coli cspD (12.4%). The deduced amino acid sequence of cspBp encoded a protein 150 residues in length, and the CspBp sequence was aligned to previously studied Csp sequences from polar bacteria: CspA from Streptomyces sp. AA8321 (CspAp), CspA from Psychromonas arctica KOPRI 22215 (CspAp) and CspAp and CspCp from P. igrنسي KOPRI 22228. All Csps have a typical β-barrel CSD composed of five β-strands, but CspBp has a unique extra domain at its N-terminal end (Fig. 1). CSD is highly conserved among many bacterial Csps, and the three-dimensional structures of some Csps, including CspAp, and Bacillus subtilis CspBp, have been reported. CSD is believed to mediate nucleic acid binding. In particular, two RNA-binding motifs, RNPI (with consensus sequence K-G-F-G-F-I) and RNPII (with consensus sequence V-F-V-H-F) are crucial for binding to RNA or ssDNA (boxed in Fig. 1). In RNPI and CspBp, the first and the second Val residues were replaced with Tyr and Thr, respectively, and the last Phe residue was replaced with Val. However, all three Phe residues (Phe-15, Phe-17, and Phe-28; residue numbers according to the canonical CspAp) in the RNA-binding motifs, which are considered to be necessary for nucleic acid-binding activity, were conserved in CspBp. Sequence analysis of CspBp suggested that the protein may bind to RNA or ssDNA through a canonical cold shock domain β-barrel structure. Meanwhile, the additional N-terminal domain of this protein did not show any noticeable homology to other proteins.

**Cold-resistance of the host overexpressing CspBp was greatly increased**

To study the roles of Csps from psychrophilic bacteria, cold resistance of the hosts harboring csp genes, was examined. Csp-overexpression was induced by the addition of IPTG to mid-log phase liquid cultures of cells carrying a csp-expression vector. When E. coli cells harboring pAED4 were frozen and thawed once, less than 1% of the original cells survived. Following repeated cycles of freezing and thawing, the number of surviving cells decreased almost exponentially. Overexpression of previously reported csp genes from polar bacteria increased freeze-survival rates of the hosts only moderately: the Ps. arctica CspAp-expressing cells exhibited a slightly increased survival rate and the CspAp or CspCp-overexpressing cells showed more than five-fold increase in the survival rates in the first freeze-thaw cycle (Fig. 2). Surprisingly, the CspBp-overexpressing cells showed an extraordinary increase in freeze-tolerance: more than fifty fold the number of cells survived the first freeze–thaw cycle and the number of surviving cells increased to greater than 100,000-fold after three cycles of freezing and thawing compared to the number of surviving pAED4-carrying cells (Fig. 2).

**CspBp binds oligo(dT)-cellulose**

Since CspBp contains CSD and the conserved Phe residues in the RNPI and RNPII sequence motifs, which suggest single-stranded RNA or DNA binding activity, the functionality of the single-stranded nucleic acid-binding motifs of this protein was examined. Upon incubation of E. coli BL21 cells harboring pAED-cspBp with 0.1 mM IPTG, a protein band with
Fig. 1 – Sequence alignment of various Csp proteins from polar organisms. Abbreviations for bacterial CspG whose amino acid sequences were analyzed here: S. sp. CspA, Streptomyces sp. AA8321 CspA;28 P. irgensii CspA and CspC, Polaribacter irgensii KOPRI 22228 CspA and CspCp, respectively;16 P. irgensii CspB, P. irgensii KOPRI 22228 CspB; from this study; P. arctica CspA, Psychromonas arctica KOPRI 22215 CspAp.21 The amino acid sequences of Csp proteins were aligned using the default settings of CLUSTAL W.27 Below the protein sequences is a key denoting conserved sequence (.), conservative mutations (.), semi-conservative mutations (.), and non-conservative mutations (J). Gaps indicated by hyphens (-) were introduced to improve alignment. The RNA-binding motifs RNP1 and RNP2 are boxed.

an apparent molecular mass of 23 kDa on SDS-PAGE was observed. CspBp was expressed in soluble form and purified by anion-exchange column chromatography. Partially purified CspBp was incubated with oligo(dT)-cellulose beads at 4 °C for 4 h and subjected to a brief centrifugation. When the reaction products were analyzed by 20% SDS-PAGE, CspBp was bound to the oligo(dT)-cellulose and co-precipitated with the resins, while neither bovine serum albumin used at the same concentration nor contaminating proteins from the CspBp preparation were co-precipitated (Fig. 3). The result suggests that CspBp binds ssDNA.

CspBp suppresses the cold-sensitive phenotype of the csp quadruple-deletion E. coli strain

Since overexpression of CspBp greatly increased the cold resistance of wild-type E. coli (Fig. 2), the ability of CspBp to suppress the cold sensitivity of the E. coli quadruple csp deletion strain BX04 (ΔcspA, ΔcspB, ΔcspG, and ΔcspE) was also examined. Mid-log phase cultures of BX04 cells harboring pAED4, pAED-cspAp, pAED-cspBp, or pAED-cspCp were streaked onto LB plates containing 0.1 mM IPTG and incubated at temperatures ranging from 16 to 37 °C. As described previously,17 the growth of BX04 cells was comparable to other clones at 37 °C, but the growth was extremely retarded at 16 °C (Fig. 4). Meanwhile, overexpression of any Csp from P. irgensii complemented the cold-sensitivity of BX04 at 25 °C and at 16 °C (Fig. 4). When the growth of BX04 was followed by OD600, overexpression of CspBp promoted the growth at low temperatures at slightly higher levels than CspAp or CspCp did (data not shown).

Discussion

Cold temperatures affect all physical-chemical parameters of living organisms, and effects include decreased solute diffusion rates, ice crystal formation, dehydration, decreased membrane fluidity, slow enzyme reaction rates, stable secondary structure formation in nucleic acids, and cold-denaturation of proteins. Psychrophilic organisms from cold ecosystems have evolved biological means to circumvent these challenges. Although transcription and translation of most genes are nearly stopped upon sudden temperature drops in mesophiles, expression of cold-shock genes are selectively induced.1 Meanwhile, corresponding genes in psychrophiles are more consistently expressed, instead of being transiently induced during the cold acclimation phase,3 suggesting that CspS play important roles for survival in cold environments.

In an effort to understand the mechanisms played by psychrophiles to adapt to cold biosphere, this study focused on the functions of CspG from polar bacteria. Although dozens of csp genes from psychrophiles have been identified by genomic/metagenomics approaches, their roles on
cold-adaptation were elucidated only in very limited cases. Heterogeneous expression of Csps from polar microorganisms resulted in various effects on cold adaption of their recombinant hosts. Certain Csps failed to increase cold-resistance of their hosts: CspAS from an Antarctic Streptomyces neither increased the cold-resistance of wild-type E. coli, nor that of the E. coli quadruple csp deletion strain BX04. Instead overproduction of CspAS inhibited DNA replication, as non-canonical E. coli CspD does, suggesting a role for CspAS in halting DNA replication until the cell adjusts itself upon sudden temperature drops. Similarly, overexpression of a Csp from an Antarctic haloarchaeon Haloarcula marismortui did not suppress the cold sensitivity of BX04. Meanwhile, it has been reported that the overexpression of CspAPa increased the cold-resistance of wild-type E. coli, but not of the E. coli quadruple csp deletion strain BX04. Therefore, the contribution of CspAPa to the cold survival of their recombinant hosts seemed relatively modest. Other Csps from psychrophilic organisms were more effective in increasing cold tolerance of their hosts; the overexpression of CspAPi or CspCPi not only increased the cold-survival rates of wild-type E. coli by more than five-fold following one cycle of freezing and thawing, but also rescued cold-sensitive phenotype of BX04.

Overexpression of Csps from a stenopsychrophilic archaeon Methanoglobus frigidus or a deep sea planktonic archaeon Crenarchaeota also complemented cold susceptibility of E. coli BX04. Since no quantitative data on freeze-tolerance conferred by these proteins were provided, more detailed studies would be necessary to compare their effect with that of CspPi. However, overexpression of any E. coli Csps, except CspDEc, also rescued cold-sensitive E. coli BX04, and overexpression of CspACe increased survival rates of the wild-type E. coli upon freezing and thawing at a comparable level to CspAPi or CspCPi (data not shown). These results show that the ability to confer cold resistance to their hosts is not the unique characteristics

Fig. 2 – Greatly increased cold-resistance of CspB_{Pi}-overexpressing cells. The survival rates of various Csp-overexpressing cells following cycles of freeze-and-thaw are shown. The number of viable cells prior to freezing was set at 100%. ●, Control pAED4-carrying cells; ▼, CspAPi-overexpressing cells; ○, CspAPa-overexpressing cells; ■, CspCPi-overexpressing cells; ▲, CspB_{Pi}-overexpressing cells.

Fig. 3 – Oligo(dT)-binding activity of CspB_{Pi}. CspB_{Pi} was incubated with oligo(dT)-cellulose at 4°C for 4 h. Proteins bound to beads were collected by centrifugation. Lanes: MW, precision plus protein standards (Bio-Rad Laboratories Inc.; size of each protein band is shown in kDa at left of the gel); BSA, bovine serum albumin; CspB, CspB_{Pi}; bead, oligo(dT)-cellulose only. The migration position of the bound CspB_{Pi} protein is indicated with a red box.

Fig. 4 – Overexpression of CspB_{Pi} rescued cold-sensitive phenotype of csp quadruple-deletion E. coli strain BX04. BX04 cells harboring pAED, pAED-cspAPi, pAED-cspB_{Pi}, or pAED-cspCPi were grown in the liquid culture to an OD_{600} of 0.4. The cells were then streaked on LB plates containing 0.1 mM IPTG, and incubated at temperatures ranging from 16 to 37°C.
of Csps from psychrophiles: previous studies rather suggest that several Csps from psychrophiles have retained sufficient similarity throughout evolution to be able to function effectively in mesophiles to confer cold tolerance.

Surprisingly, overexpression of CspBγ greatly increased the cold tolerance of its recombinant host to an unprecedented level (Fig. 2). Overexpression of CspBγ not only induced a more than fifty fold increase in freeze-tolerance in wild-type E. coli (Fig. 2), but also noticeably promoted the growth of the E. coli quadruple csp deletion strain at low temperatures (Fig. 4). Elucidating the detailed mechanisms involved in freeze-tolerance conferred by CspBγ would be of great academic interest and will be pursued in a future study. CspBγ possesses cold shock domains at the C-terminal region (Fig. 1) and shares the basic characteristics of Csps, including the ability to bind single-stranded nucleic acids, as indicated by oligo(dT)-binding assays (Fig. 3). One possibility is that the CSD of CspBγ may have greatly improved activity, for example as a RNA chaperone, maintaining single stranded nucleic acid structures at extremely low temperatures and allowing efficient transcription and translation. On the other hand, CspBγ has an extra domain on its N-terminal region, and it is also possible that this unique region plays a distinct role in conferring an extraordinary increase in cold survival ability. Sequence homology search has identified several cspBγ-homologous genes, encoding both N-terminal extra domain and CSD, from evolutionarily related marine Flavobacteriaceae, including Polaribacter sp. MED152, Lacinutrix sp. SH-3-7-4, Winogradskyella sp. PC-2, and Maribacter sp. HTCC2170. However, their functional roles on cold tolerance or transcriptional/translational regulation upon temperature downshifts have not been studied yet. It will be interesting to test freeze-tolerance of hosts overexpressing these dual domain Csp-like proteins.

Introduction of CspBγ into other organisms has potential industrial applications, including increased cold-resistance of nitrogen-fixing bacteria, such as Rhizobium and cyanobacteria, and the survival of starter cultures after storage at freezing temperatures. The long-pursued development of frozen dough may be achievable by improving the freeze-tolerance of baker’s yeast. The possible application of CspBγ does not need to be limited to industrially important microorganisms and it may be introduced into plants to enhance their viability at low temperatures and increase their economic value.

**Conflicts of interest**

The authors declare no conflicts of interest.

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