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Rescue of a Cold-Sensitive Mutant at Low Temperatures by Cold Shock Proteins from *Polaribacter irgensii* KOPRI 22228

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Exposure to low temperatures induces the biosynthesis of specific sets of proteins, including cold shock proteins (Csps). Since many of the specific functions of pychrophilic Csps are unknown, the roles of Csps from an Arctic bacterium, *Polaribacter irgensii* KOPRI 22228, were examined. The genes encoding CspA and CspC of *P. irgensii* were cloned in this study. Sequence analysis showed that these proteins have cold shock domains containing two RNA-binding motifs, RNP1 and RNP2. Both proteins bound oligo(dT)-cellulose resins, suggesting single-stranded nucleic acid-binding activity. When the *P. irgensii* Csps were overexpressed in *Escherichia coli*, the cold-resistance of the host was increased by more than five-fold. The *P. irgensii* Csps also rescued a cold-sensitive *E. coli csp*-quadruple deletion strain, BX04, at low temperatures. These results suggest that Csps from *P. irgensii* play a role in survival in polar environments.

***Keywords*:** cold-shock protein (Csp), psychrophile, Arctic bacteria, cold-resistance, *P. irgensii*

Cold stress is a major environmental challenge for organisms. Low temperatures can cause several serious problems in living organisms, including decreased membrane fluidity, which can hamper vital membrane functions such as transport and protein movement; the formation of stable secondary structures in DNA and RNA, which can inhibit transcription and translation; the association of an inhibitory factor with ribosomes; increased DNA super-coiling, which may hinder genetic information processing; slow protein folding, leading to protein misfolding; and reduced enzymatic reaction rates, which can limit cellular metabolism (Jones *et al*., 1987; Phadtare, 2004; D'Amico *et al*., 2006). In particular, at the North and South Poles, temperature downshifts are a major environmental challenge for bacteria. Despite the potential for problems, relatively high numbers of diverse microorganisms survive in the Polar Regions; for example, 10^5-10^6 cells/ml have been found even in Arctic and Antarctic pack ice (Brinkmeyer *et al*., 2003). Thus, microorganisms living in Polar Regions must possess distinct survival mechanisms that enable them to survive in extremely cold environments.

Although the biosynthesis of most cellular proteins is repressed upon a sudden drop in temperature, some sets of proteins, including cold shock proteins (Csps), are induced (Jones *et al*., 1992). Csp homologs have been found in a wide range of bacteria (Ermolenko and Makhatadze, 2002), including the mesophilic bacterium *Escherichia coli* (Goldstein *et al*., 1990); the psychrotrophic bacteria *Streptomyces* sp. *AA8321* (Kim *et al*., 2007), *Pseudomonas fragi* (Michel *et al*., 1997), and *Pseudomonas putida* (Gumley and Inniss, 1996); the psychrophilic bacterium *Psychromonas artica* KOPRI 22215 (Jung *et al*., 2010); the thermophilic bacterium *Thermotoga maritime* (Phadtare *et al*., 2003); and the archaeons *Methanogenium frigidum* and *Halorubrum lacusprofundi* (Giaquinto *et al*., 2007).

Following a temperature downshift from 37 to 10°C, CspA of *E. coli* ($CspA_{Ec}$), a major cold-shock protein, was induced more than 200-fold, reaching a cellular concentration of 100 μM within 1.5 h (Jones and Inouye, 1994). $CspA_{Ec}$ binds single-stranded DNA (ssDNA) and RNA, but not double-stranded DNA. $CspA_{Ec}$ is believed to function as an RNA chaperone (Jiang *et al*., 1997), melting the stable secondary structures in RNA molecules, which facilitates translation at low temperatures (Jones *et al*., 1992; Schindler *et al*., 1999). RNA chaperones may also regulate the rate of mRNA degradation and transcription termination (Ermolenko and Makhatadze, 2002). Eight other Csps (from CspB to CspI) have been reported in *E. coli* (Ermolenko and Makhatadze, 2002). Among these, only CspB, CspG, and CspI are induced by cold shock (Ermolenko and Makhatadze, 2002). The deletion of one, two, or three cold-inducible *E. coli csp* genes does not affect cell viability at low temperatures, suggesting their redundant functions. A quadruple *csp* deletion mutant (Δ*cspA*, Δ*cspB*, Δ*cspG*, and Δ*cspE*) of *E. coli* exhibited increased cold sensitivity (Xia *et al.*, 2001); however, the cold-sensitive phenotype of this strain could be suppressed by the overexpression of any Csp homolog except CspD (Xia *et al.*, 2001). CspD of *E. coli* is not induced by cold; instead, it is induced during the stationary phase and upon nutrient starvation. Moreover, CspD of *E. coli* and CspA of *Streptomyces* sp. AA8321 can inhibit DNA replication (Yamanaka *et al*., 2001; Kim *et al*., 2007).

The three-dimensional structures of some Csps, including $CspA_{Ec}$ and CspB from *Bacillus subtilis*, have been reported (Schindelin *et al.*, 1993; Newkirk *et al*., 1994). All known Csps contain a β-barrel structure known as a cold shock domain, which is believed to mediate nucleic acid binding. Two RNA-

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binding motifs, RNP1 and RNP2, are particularly critical for the nucleic-acid binding ability of Csps (Schröder *et al*., 1995). However, most functional studies have been done on Csps from mesophilic bacteria, like *E. coli*, rather than on Csps from psychrophilic bacteria.

To understand the mechanism of survival for micro-organisms in Polar Regions, a psychrophilic bacterium from Arctic sea sediment was isolated and identified as *Polaribacter irgensii*. Searches for *csp* genes in *P. irgensii* genomic sequences have identified three candidate genes. One of them exhibited low homology to the canonical *E. coli* cspA (15.8%) and cspD (12.4%), and it was subjected to a future study. Two other *csp* genes from *P. irgensii* KOPRI 22228, $cspA_{Pi}$ and $cspC_{Pi}$, exhibiting considerable homology to other *csps*, were cloned in this study, and functional and phylogenic analyses of the Csps were performed.

Materials and Methods

Bacterial culture and species identification

Microbial samples were collected from Arctic sea sediments near Dasan Korean Arctic Station (Ny-Alesund, Norway) and inoculated on solid Zobell plates (0.4% peptone, 0.1% yeast extract, 1.5% agar in artificial seawater prepared with 2.3% NaCl, 20 mM KCl, 5 mM $MgSO_4$, and 2 mM $NaCl_2$). The species of isolated colonies were determined by 16S rDNA sequences, as described (Jung *et al*., 2010). The isolated single colonies were re-streaked and incubated at various temperatures ranging from 4 to 37°C for the analysis of temperature-dependant growth patterns.

Cloning of *csp* genes from *P. irgensii* KOPRI 22228

Two *csp* genes were obtained by PCR amplification. The template DNA was extracted from *P. irgensii* KOPRI 22228 cells using G-spin™ bacteria genomic DNA Extraction kit (Intron Co., Korea), according to the protocol suggested by the manufacturer. The forward primer sequences were $CspA_{Pi}$F1N (5′-TTATAACCATATGAGTAAAGGTAC AGTAAAGTTTTTCA-3′), and $CspC_{Pi}$F1N (5′-CTTTATTCATATGA ATAAAGGTACCGTAAAATTTTTC AATG-3′). The backward primers were $CspA_{Pi}$B63*B (5′-CGGGATCCTTAGATAACTTTTACGTTAA CTGCATTTAAT-3′), and $CspC_{Pi}$B63*B (5'-CTGGATCCTTATATAA CTCTTACGTTTACTGCGTTT-3′) (manufactured by Bioneer Co., Korea). The PCR mixture consisted of 5 μl of 10× PCR buffer (final concentrations: 50 mM KCl, 0.01% gelatin, 10 mM Tris-HCl, pH 9.0), 2.5 mM $MgCl_2$, 0.2 mM for each dNTP, 200 nM for 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 53-56°C for 30 sec, and extension at 72°C for 1 min. A final extension was performed at 72°C for 5 min. The PCR products that were 0.2 kb in size were double-digested with *Nde*I and *Bam*HI, and cloned using an *E. coli* expression vector (pAED4; Jung and Im, 2003) 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 were named pAED-$cspA_{Pi}$ and pAED-$cspC_{Pi}$, respectively.

Expression of Csps in *E. coli*

The recombinant pAED-$cspA_{Pi}$ and pAED-$cspC_{Pi}$ plasmids were transformed into competent *E. coli* BL21(DE3) cells (Invitrogen Co., USA) separately. For overproduction of Csps, 1 ml of the overnight liquid culture was transferred to a flask containing 50 ml fresh LB medium containing 100 μg/ml ampicillin. Cells were grown in liquid culture to an OD_{600} of 0.4, and IPTG was added to the final concentration of 0.1 mM. The cultures were incubated further at 37°C for various times with vigorous shaking. Overexpression of the Csps was analyzed by 20% SDS-PAGE. The protein bands were visualized by Coomassie brilliant blue R250 staining.

Purification of $CspA_{Pi}$ and $CspC_{Pi}$

E. coli BL21(DE3) cells overexpressing either $CspA_{Pi}$ or $CspC_{Pi}$ were harvested from 1 L culture by centrifugation, and resuspended in 40 ml of 10 mM phosphate buffer, pH 6.5. Cells were lyzed by sonication using a Bandelin Sonoplus HD2200 ultrasonic homogenizer (Germany) as described (Kim *et al*., 2007). The supernatant fraction was loaded on a Q-sepharose™ (Amersham Bioscience Co.) fast flow ion exchange column equilibrated with the same buffer. Csps were eluted with a 0-0.5 M NaCl gradient, and size-fractionated through a Centricon (Viva Science Technology Co., Germany) with a molecular cut-off of 30 kDa. Filtrates were collected, and the purity of proteins was analyzed by 20% SDS-PAGE. Concentrations of proteins were determined using Bio-Rad DC (detergent compatible) protein assay kit.

Oligo(dT)-cellulose binding assays

Purified Csps were dialyzed against binding buffer (10 mM Tris-HCl; pH 8.0, 1 mM EDTA, 50 mM KCl, and 7.4% glycerol). Fifty microliter of oligo(dT)-cellulose type 7 beads (Amersham Bioscience Co.) were incubated with 50 μg of Csp proteins at 4°C for 4 h. The resins and bound proteins were collected by brief centrifugation, and washed twice with the binding buffer. Co-precipitated proteins were analyzed by 20% SDS-PAGE and Coomassie brilliant blue R250 staining.

Resistance to freezing and thawing

Csp overexpression was induced by the addition of IPTG to mid-log phase liquid cultures of cells carrying either pAED-$cspA_{Pi}$ or pAED-$cspC_{Pi}$ at 37°C for 2 h. Then, 1 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 repeated up to three times. Colony-forming units (CFU) were analyzed at each cycle of freeze-and-thaw. The data were collected from five independent experiments and shown as an average with standard error calculations for each point.

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

E. coli quadruple *csp* deletion strain BX04 cells harboring pAED, pAED-$cspA_{Pi}$, or pAED-$cspC_{Pi}$ were grown in the liquid culture to an OD_{600} of 0.4. The cells were then streaked onto LB plates containing 0.1 mM IPTG, and incubated at temperatures ranging from 15 to 37°C. After 16 h at 37°C, 32 h at 25°C, and 72 h at 15°C, growth of BX04 cells on the plates were observed.

Results

Cloning of the *csp* genes from *P. irgensii* KOPRI 22228

Microbial samples were collected from Arctic sea sediments near Dasan Korean Arctic Station (Ny-Alesund, Norway). One of the isolated microorganisms was identified as *P. irgensii* KOPRI 22228 by phylogenic analysis of its 16S rDNA sequence. This bacterium grew optimally at 10°C; the maximum growth

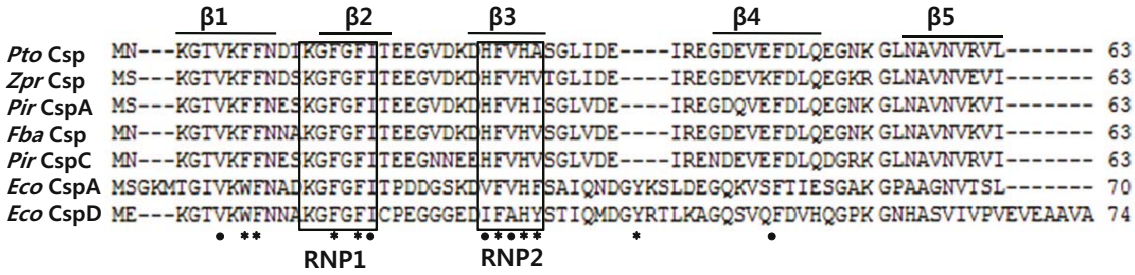

Fig. 1. Sequence alignment of $CspA_{Pi}$ and $CspC_{Pi}$ proteins with other representative bacterial Csp proteins. The Csp sequences were obtained from the GenBank database and aligned with the default settings of CLUSTAL W (Thompson *et al.,* 1994). The bacterial strains from which Csp amino acid sequences analyzed in this study were extracted: *Pto, Psychroflexus torquis* ATCC 700755; *Zpr*, *Zunongwangia profunda* SM-A87; *Pir*, *P. irgensii*; *Fba*, *Flavobacteriales bacterium* HTCC2170; and *Eco*, *E. coli.* Gaps indicated by hyphens (-) were introduced to improve alignment. The RNA-binding motifs RNP1 and RNP2 are boxed. Eight aromatic residues that are critical for binding RNA or ssDNA are indicated with asterisks (*), and five hydrophobic residues forming the hydrophobic core are indicated with solid circles (●) on the lowest line. The five β-strands (β1 to β5) are shown on the top line.

temperature was 25°C, suggesting that the bacterium is a psychrophile.

Since the roles of Csps from psychrophilic bacteria have not been assessed in detail, we decided to study the roles of the Csps from *P. irgensii* KOPRI 22228. DNA fragments encoding the Csps were amplified by PCR, based on the genomic sequence of *P. irgensii* ATCC 700398. Those products that were 0.2 kb in size were then cloned using an *E. coli* expression vector (pAED4). The nucleotide sequences of $cspA_{Pi}$ and $cspC_{Pi}$ contained ORFs comprised of 189 nucleotides. A BLAST nucleotide homology search identified *csp* genes from other bacteria. $cspA_{Pi}$ was 85% homologous to a *csp* from *Flavobacteriales bacterium* HTCC2170, 79% to that from *Zunongwangia profunda* SM-A87, and 77% to that from *Croceibacter atlanticus* HTCC2559; in comparison, $cspC_{Pi}$ was 83% homologous to a *csp* from *F. bacterium* HTCC2170, 81% to that from *Z. profunda* SM-A87, and 78% to that from *C. atlanticus* HTCC2559.

Primary sequence analysis of *P. irgensii* Csps

The deduced amino acid sequences of $cspA_{Pi}$ and $cspC_{Pi}$ each encoded a low-molecular-weight acidic protein, 63 residues in length, with typical β-barrel cold shock domains composed of five β-strands (indicated by straight lines above the sequences in Fig. 1). Highly conserved RNA-binding motifs, RNP1 (K-G-F-G-F-I) and RNP2 (V-F-V-H-F), which are involved in binding to RNA or ssDNA, were also identified (boxed in Fig. 1); however, the first Val residue in RNP2 was replaced with His in both proteins, while the last Phe residue was replaced with Ile in $CspA_{Pi}$ and Val in $CspC_{Pi}$. Eight aromatic residues (Trp-11, Phe-12, Phe-18, Phe-20, Phe-31, His-33, Phe-34, and Tyr-42) known to be responsible for the interaction with RNA or ssDNA (Newkirk *et al*., 1994) are indicated by asterisks in Fig. 1. All three Phe residues (Phe-18, Phe-20, and Phe-31) in the RNA-binding motifs, which are considered to be necessary for nucleic acid-binding activity (Schröder *et al*., 1995; Hillier *et al*., 1998), were conserved in the Csps. The X-ray crystal structure of $CspA_{Ec}$ shows that key hydrophobic residues (Val-9, Ile-21, Val-30, Val-32, and Val-51; indicated by dots in Fig. 1) form the hydrophobic core of the β-barrel structure (Feng *et al*., 1998). These hydrophobic residues were also conserved, except for Val-30, which was replaced by His. These features of the *P. irgensii* Csp sequences suggest that the proteins bind to RNA or ssDNA through a canonical cold shock domain β-barrel structure.

At the protein level, $CspA_{Pi}$ and $CspC_{Pi}$ exhibited significant homology to a Csp from *F. bacterium* HTCC2170 (92 and 84%, respectively), *Psychroflexus torquis* ATCC 700755 (87 and 81%, respectively), and *Z. profunda* SM-A87 (86 and 78%, respectively). $CspA_{Pi}$ and $CspC_{Pi}$ also exhibited considerable homology to the canonical *E. coli* Csps $CspA_{Ec}$ (40 and 36%, respectively) and $CspD_{Ec}$ (39 and 38%, respectively).

Purified $CspA_{Pi}$ and $CspC_{Pi}$ bind oligo(dT)-cellulose

Following the high-speed centrifugation of cellular extracts from $CspA_{Pi}$- or $CspC_{Pi}$-overexpressing cells, most, if not all, of the Csps were found in the supernatant. The Csps were purified by anion-exchange column chromatography and size-fractionation as described in the Materials and Methods. A single protein band with an apparent molecular mass of 7 kDa was observed by SDS-PAGE and Coomassie Brilliant Blue staining (Fig. 2).

Since the RNP-1 and -2 sequence motifs and five hydrophobic residues implicated in the formation of a hydrophobic

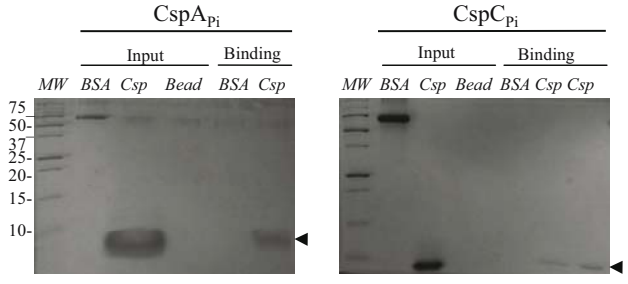

Fig. 2. Oligo(dT)-binding activity of $CspA_{Pi}$ and $CspC_{Pi}$. *P. irgensii* Csps were incubated with oligo(dT)-cellulose at 4°C for 4 h. Lanes: MW, Precision plus protein standards (Bio-Rad Laboratories Inc.; size of each protein band is shown in kDa at left of the gel); BSA, bovine serum albumin; Csp, either $CspA_{Pi}$ or $CspC_{Pi}$; and Bead, oligo(dT)-cellulose only. The migration position of the Csp protein is indicated by an arrowhead.

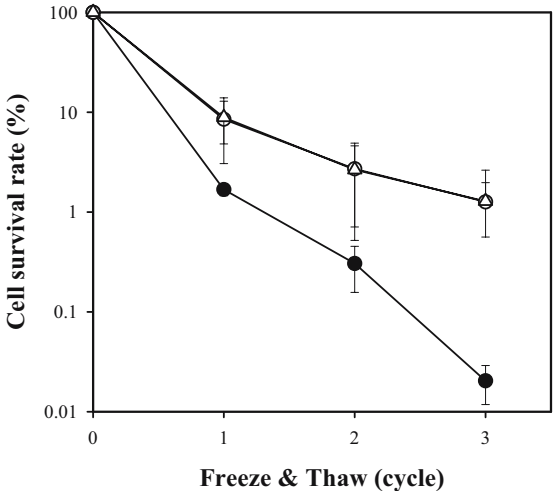

Fig. 3. Increased cold-resistance of *P. irgensii* Csp-overexpressing cells. The survival rates of Csp-overexpressing cells following cycles of freezing and thawing are shown. The number of viable cells prior to freezing was set at 100%. (●), control pAED4-carrying cells; (○), $CspA_{Pi}$-overexpressing cells; (Δ), $CspC_{Pi}$-overexpressing cells.

core were conserved in $CspA_{Pi}$ and $CspC_{Pi}$, we examined the single-stranded nucleic acid-binding ability of these proteins using purified Csps that were incubated with oligo(dT)-cellulose beads at 4°C for 4 h in binding buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM KCl, and 7.4% glycerol). When the reaction products were separated by 20% SDS-PAGE, $CspA_{Pi}$ and $CspC_{Pi}$ were bound to the oligo(dT)-cellulose and co-precipitated with the resins, while bovine serum albumin used at the same concentration was not (Fig. 2). Thus, $CspA_{Pi}$ and $CspC_{Pi}$ bind ssDNA.

Overexpression of *P. irgensii* Csps increases the cold-resistance of the host

To study the roles of Csps from psychrophilic bacteria, cold resistance of the host harboring *cspA* or *cspC* from *P. irgensii* was examined (Fig. 3). Csp overexpression was induced by the addition of IPTG to mid-log phase liquid cultures of cells carrying either pAED-$cspA_{Pi}$ or pAED-$cspC_{Pi}$. When *E. coli* cells harboring pAED4 were frozen and thawed once, less than 2% of the original cells survived and were able to form colonies. Following repeated cycles of freezing and thawing, the number of surviving cells decreased almost exponentially. Meanwhile, more than five-fold of the Csp-overexpressing cells survived the first freeze-thaw cycle. After three cycles of freezing and thawing, the number of surviving Csp-overproducing cells was more than 60-fold greater, compared to the number of surviving pAED4-carrying cells.

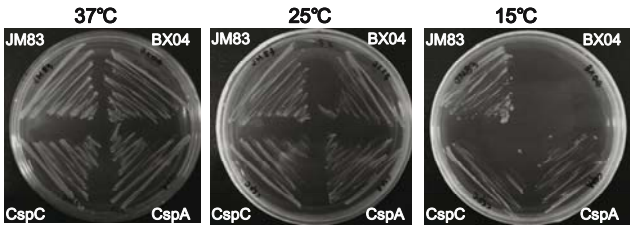

Fig. 4. *P. irgensii* Csp proteins suppress cold-sensitive phenotype of *csp* quadruple-deletion *E. coli* strain, BX04. BX04 cells harboring pAED4, pAED-$cspA_{Pi}$, or pAED-$cspC_{Pi}$ were grown in the liquid culture to an OD_{600} of 0.4. The cells were then streaked on LB plates containing 0.1 mM IPTG, and incubated at temperatures ranging from 15 to 37°C. *E. coli* JM83 strain was used as wild-type control.

P. irgensii Csps suppress the cold-sensitive phenotype of the *csp* quadruple-deletion *E. coli* strain, BX04

Since overexpression of the *P. irgensii* Csps increased the cold resistance of wild-type *E. coli* (Fig. 3), we tested whether the Csps could complement the cold-sensitivity of the *E. coli* quadruple *csp* deletion strain BX04 (Δ*cspA*, Δ*cspB*, Δ*cspG*, and Δ*cspE*). BX04 cells harboring pAED4, pAED-$cspA_{Pi}$, or pAED-$cspC_{Pi}$ were grown in liquid culture to an OD_{600} of 0.4. The cells were then streaked onto LB plates containing 0.1 mM IPTG and incubated at temperatures ranging from 15-37°C. As described previously (Xia *et al.*, 2001), BX04 cells were able to form colonies at 37°C, but not at 15°C, whereas the wild-type *E. coli* strain JM83 formed colonies at all temperatures tested. The *P. irgensii* Csps complemented the cold-sensitivity of BX04, allowing its growth at 15°C (Fig. 4).

Discussion

The extremely cold temperatures at the North and South Poles pose several serious challenges to living organisms: ice crystal formation, dehydration caused by freezing, decreased membrane fluidity, secondary structure formation in nucleic acids, and slow protein folding. Thus, biological mechanisms for survival at extremely cold temperatures are required for organisms living in Polar Regions. Among cold-induced proteins, small acidic proteins called Csps are the most prominent. However, most functional studies have focused on Csps from the mesophilic bacterium *E. coli*, and not on those from psychrophilic bacteria. Increasing numbers of Csp homologs have been reported from psychrophiles, mostly through genomic DNA sequencing projects, but little functional data are available.

In an effort to understand the molecular mechanisms of psychrophilic bacteria that allow them withstand freezing environments, we previously studied the role of a Csp from *Psychromonas arctica* KOPRI 22215 ($CspA_{Pa}$), which was obtained from Arctic sea sediment (Jung *et al*., 2010). The overexpression of $CspA_{Pa}$ in *E. coli* increased the cold-resistance of the wild-type host, but not of the *E. coli* quadruple *csp* deletion strain BX04. The phenotypes of $CspA_{Pa}$-overexpressing cells have a precedent; the overexpression of *E. coli* $CspD_{Ec}$ could not complement the cold-sensitive phenotype of BX04, whereas overexpression of $CspD_{Ec}$ increased the cold-resistance of the wild-type strain (Xia *et al.*, 2001). The authors interpreted this result to mean that the overproduction of $CspD_{Ec}$ is toxic to the host (Yamanaka *et al.*, 2001). Indeed, the overexpression of $CspA_{Pa}$ impaired colony formation and made cellular shape elongated, suggesting that $CspA_{Pa}$ does not complement the cold-sensitivity of BX04 due to its toxicity (Jung *et al.*, 2010).

Therefore, we examined other Csps from a psychrophile: $CspA_{Pi}$ and $CspC_{Pi}$. Sequence comparisons to the well-characterized proteins $CspA_{Ec}$ and $CspD_{Ec}$ revealed that the

level of homology to the *E. coli* Csps was reduced in the *P. irgensii* Csps (36-40%) compared to *P. arctica* CspA (56%), indicating the unique characteristics of the *P. irgensii* Csps. The *P. irgensii* Csps share the basic characteristics of Csps, including the ability to bind single-stranded nucleic acids, as indicated by oligo(dT)-binding assays (Fig. 2). Sequence analysis also suggests the presence of cold shock domains in the *P. irgensii* Csps (Fig. 1). Since the overexpression of $CspA_{Pi}$ or $CspC_{Pi}$ increased the cold-resistance of the host by more than five-fold following one cycle of freezing and thawing (Fig. 3), our experiments suggest a role for *P. irgensii* Csps in survival in Polar Regions, where organisms are likely to encounter repeated freeze-thaw conditions. Furthermore, $CspA_{Pi}$ and $CspC_{Pi}$ complemented the cold-sensitivity of the *E. coli* quadruple *csp* deletion strain BX04 (Fig. 4). This result suggests a possible academic and industrial application, since cold resistance is a critical factor in the survival of starter cultures after storage at freezing temperatures (Willimsky *et al*., 1992; Wouters *et al*., 2001). Our results suggest that the introduction of *P. irgensii csp* genes into industrially important microorganisms, and possibly into plants, may enhance their viability at low temperatures and increase their economic value.

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