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CspB of an arctic bacterium, *Polaribacter irgensii* 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 irgensii* 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. irgensii* CspB bound to oligo(dT)-cellulose resins, suggesting single-stranded nucleic acid-binding activity. The unprecedented level of freeze-tolerance conferred by *P. irgensii* 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

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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.⁴ 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.⁵ 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,⁶ melting the stable secondary structures in RNA molecules, which facilitates translation and ribosome biogenesis.^{7–9} Several chaperones, such as GroEL¹⁰ and DnaK,¹¹ 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 isomerases play an important role in cold adaptation by facilitating the folding of functionally significant proteins.¹² Cryoprotectants, such as antifreeze proteins,¹³ trehalose,² and exopolysaccharides,¹⁴ 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.¹⁵

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.¹⁶ Two *csp* genes, *cspA_{pi}* and *cspC_{pi}*, were previously cloned from this bacterium and exhibited considerable homology to canonical *Escherichia coli* *cspA* (*CspA_{Ec}*). 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 (Δ *cspA*, Δ *cspB*, Δ *cspG*, and Δ *cspE*) strain,¹⁷ suggesting functional homology among those Csp-family proteins.¹⁶ In this study, we report another Csp-homologous protein from *P. irgensii* KOPRI 22228, *CspB_{pi}*, 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 *cspB_{pi}* gene

P. irgensii KOPRI 22228 was isolated from Arctic Sea sediments near Dasan Korean Arctic Station (Ny-Alesund, Norway), and cultured as previously reported.¹⁶ The *cspB_{pi}* 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 (Intron Co., Korea), according to the protocol suggested by the manufacturer. The forward primer sequence was *CspB_{pi}* F1H, 5'-AGTAAGCTTATGGCAAAATCGCAGCAGACCT-3', and the reverse primer was *CspB_{pi}* B150*B, 5'-CCGGATCCTTATATTTGGTAACCTTAACTGCATTGATT-3' (manufactured by Bioneer Co., Daejeon, Korea). The PCR mixture consisted of 5 μ l of 10 $\times$ PCR buffer (final concentrations: 50 mM KCl, 0.01% gelatin, 10 mM Tris-HCl, pH 9.0), 2.5 mM MgCl₂, 0.2 mM of each dNTP, 200 nM of each primer, 1 μ l of template DNA, and 2.5 units of Taq DNA polymerase (Takara, Japan) in the final 50 μ l 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 pAED4,¹⁸ 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 were named pAED-*cspB_{pi}*.

Expression of *CspB_{pi}* in *E. coli*

The recombinant plasmid for expression of *CspB_{pi}*, pAED-*cspB_{pi}*, was transformed into competent *E. coli* BL21(DE3) cells (Invitrogen Co., CA, USA) using the method described by Sambrook et al.¹⁹ For overproduction of *CspB_{pi}*, 1 ml of the overnight liquid culture was transferred to a flask containing 50 ml fresh LB medium containing 100 μ g/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 *CspB_{pi}* was analyzed by 20% SDS-PAGE. The protein bands were visualized by Coomassie brilliant blue R250 staining.

Resistance to freezing and thawing

CspB_{pi} protein was overexpressed in *E. coli* as described above. As the experimental control, *E. coli* transformed with a pAED4 plasmid lacking the *cspB_{pi}* 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 CspB_{Pi}

CspB_{Pi} 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 CspA_{Pi}.¹⁶ The supernatant fraction was loaded on a Q-sepharose™ (Amersham Bioscience Co.) fast flow ion exchange column equilibrated with the same buffer. CspB_{Pi} 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 CspB_{Pi} 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 CspB_{Pi} 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 CspB_{Pi} 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,¹⁸ pAED-cspA_{Pi},¹⁶ pAED-cspB_{Pi}, or pAED-cspC_{Pi}¹⁶ 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. irgensii* KOPRI 22228

P. irgensii KOPRI 22228 was isolated from Arctic Sea sediments and grown as previously reported.¹⁶ This bacterium is a psychrophile, with an optimum growth temperature of 10 °C. In an effort to elucidate the roles played by Csps in psychrophilic bacteria, DNA fragments from this bacterium, which contain a region encoding a conserved CSD, were cloned. A new *csp*-homologous gene 0.45 kb in size was obtained and named cspB_{Pi}.

A BLAST nucleotide homology search of cspB_{Pi} was performed: cspB_{Pi} exhibited low homology to the canonical cspA_{Ec}

(15.8%) and *E. coli* cspD (12.4%). The deduced amino acid sequence of cspB_{Pi} encoded a protein 150 residues in length, and the CspB_{Pi} sequence was aligned to previously studied Csp sequences from polar bacteria: CspA from *Streptomyces* sp. AA8321 (CspA_{St}),²⁰ CspA from *Psychromonas arctica* KOPRI 22215 (CspA_{Pa})²¹ and CspA_{Pi} and CspC_{Pi} from *P. irgensii* KOPRI 22228.¹⁶ All Csps have a typical β-barrel CSD composed of five β-strands, but CspB_{Pi} 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 CspA_{Ec} and *Bacillus subtilis* CspB, have been reported.^{22,23} CSD is believed to mediate nucleic acid binding. In particular, two RNA-binding motifs, RNP1 (with consensus sequence K-G-F-G-F-I) and RNP2 (with consensus sequence V-F-V-H-F) are crucial for binding to RNA or ssDNA (boxed in Fig. 1).²⁴ In RNP2 of CspB_{Pi}, 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 CspA_{Ec}) in the RNA-binding motifs, which are considered to be necessary for nucleic acid-binding activity,^{24,25} were conserved in CspB_{Pi}. Sequence analysis of CspB_{Pi} 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 CspB_{Pi} 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* CspA_{Pa}-expressing cells exhibited a slightly increased survival rate and the CspA_{Pi} or CspC_{Pi}-overexpressing cells showed more than five-fold increase in the survival rates in the first freeze-thaw cycle (Fig. 2).^{16,21} Surprisingly, the CspB_{Pi}-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).

CspB_{Pi} binds oligo(dT)-cellulose

Since CspB_{Pi} contains CSD and the conserved Phe residues in the RNP1 and RNP2 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-cspB_{Pi} with 0.1 mM IPTG, a protein band with

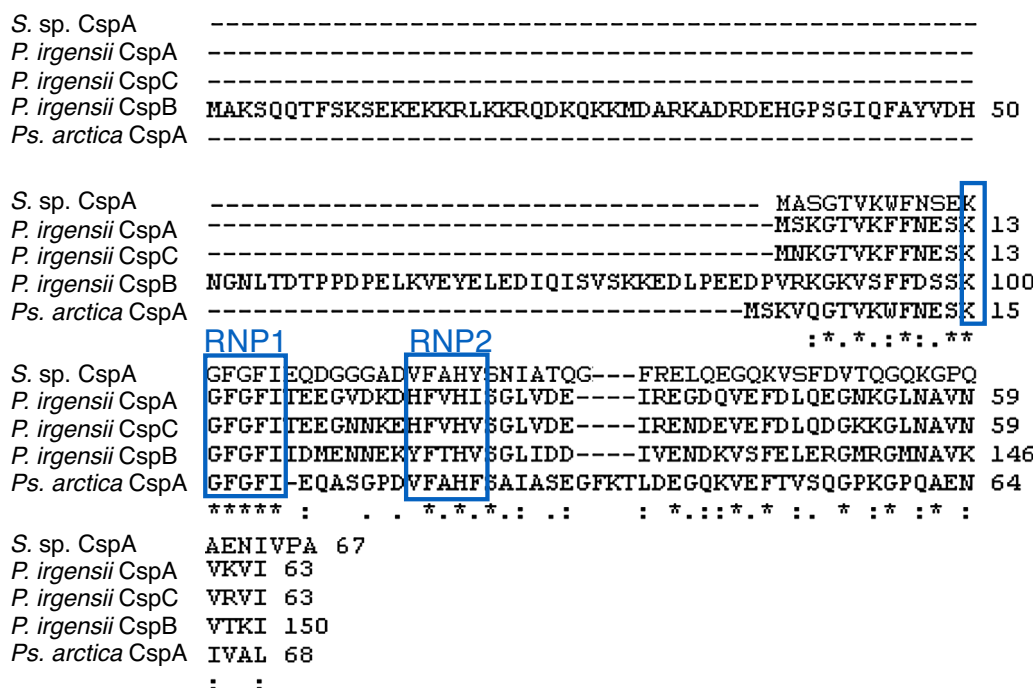


Fig. 1 – Sequence alignment of various Csp proteins from polar organisms. Abbreviations for bacterial Csps whose amino acid sequences were analyzed here: S. sp. CspA, *Streptomyces* sp. AA8321 CspA_{St}²⁰; P. irgensii CspA and CspC, *Polaribacter irgensii* KOPRI 22228 CspA_{Pi} and CspC_{Pi}, respectively¹⁶; P. irgensii CspB, P. irgensii KOPRI 22228 CspB_{Pi} from this study; Ps. arctica CspA, *Psychromonas arctica* KOPRI 22215 CspA_{Pa}.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 (). 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. CspB_{Pi} was expressed in soluble form and purified by anion-exchange column chromatography. Partially purified CspB_{Pi} 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, CspB_{Pi} 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 CspB_{Pi} preparation were co-precipitated (Fig. 3). The result suggests that CspB_{Pi} binds ssDNA.

CspB_{Pi} suppresses the cold-sensitive phenotype of the csp quadruple-deletion *E. coli* strain

Since overexpression of CspB_{Pi} greatly increased the cold resistance of wild-type *E. coli* (Fig. 2), the ability of CspB_{Pi} to suppress the cold-sensitivity of the *E. coli* quadruple csp deletion strain BX04 (Δ cspA, Δ cspB, Δ cspG, and Δ cspE) was also examined. Mid-log phase cultures of BX04 cells harboring pAED4, pAED-cspA_{Pi}, pAED-cspB_{Pi}, or pAED-cspC_{Pi} 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 Csps 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 CspB_{Pi} promoted the growth at low temperatures at slightly higher levels than CspA_{Pi} or CspC_{Pi} 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 Csps from polar bacteria. Although dozens of csp genes from psychrophiles have been identified by genomic/metagenomics approaches, their roles on

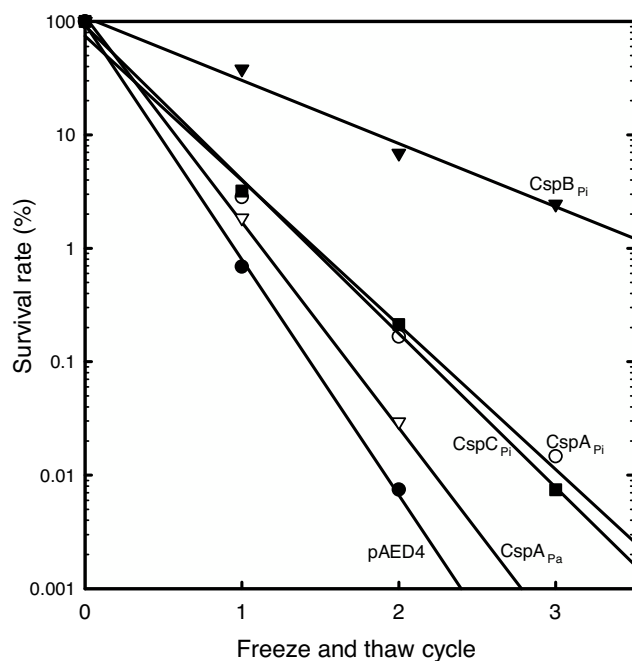


Fig. 2 – Greatly increased cold-resistance of CspB_{PI}-overexpressing cells. The survival rates of various Csp-overexpressing cells following cycles of freeze-and-thaw are shown. The number of viable cells prior to freezing was set at 100%. ●, Control pAED4-carrying cells; ▽, CspA_{Pa}-overexpressing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

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: CspA_{St} from an Antarctic *Streptomyces* neither increased the cold-resistance of wild-type *E. coli*, nor that of the *E. coli* quadruple csp deletion strain BX04.²⁰ Instead overproduction of CspA_{St} inhibited DNA replication, as non-canonical *E. coli* CspD does, suggesting a role for CspA_{St} in halting DNA replication until the cell adjusts itself upon sudden temperature drops.²⁰ Similarly, overexpression of a Csp from an Antarctic haloarchaeon *Halorubrum lacusprofundi* did not suppress the cold sensitivity of BX04.²⁶ Meanwhile, it

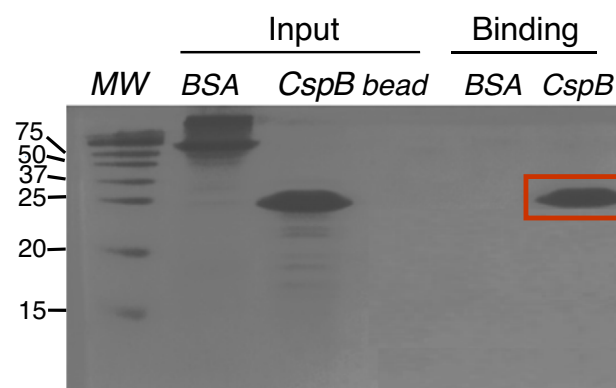


Fig. 3 – Oligo(dT)-binding activity of CspB_{PI}. CspB_{PI} was incubated with oligo(dT)-cellulose at 4 °C for 4 h. Proteins bound to beads were collected by centrifugation. Lanes: MW, precision plus protein standards (Bio-Rad Laboratories Inc.; size of each protein band is shown in kDa at left of the 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.

has been reported that the overexpression of CspA_{Pa} increased the cold-resistance of wild-type *E. coli*, but not of the *E. coli* quadruple csp deletion strain BX04.²¹ Therefore, the contribution of CspA_{Pa} 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 CspA_{PI} or CspC_{PI} 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 BX04.¹⁶ Overexpression of Csps from a stenopsychrophilic archaeon *Methanogenium frigidum* or a deep sea planktonic archaeon *Crenarchaeota* also complemented cold susceptibility of *E. coli* BX04.²⁶ 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 CspB_{PI}. However, overexpression of any *E. coli* Csps, except CspD_{EC}, also rescued cold-sensitive *E. coli* BX04,¹⁷ and overexpression of CspA_{EC} increased survival rates of the wild-type *E. coli* upon freezing and thawing at a comparable level to CspA_{PI} or CspC_{PI} (data not shown). These results show that the ability to confer cold resistance to their hosts is not the unique characteristics

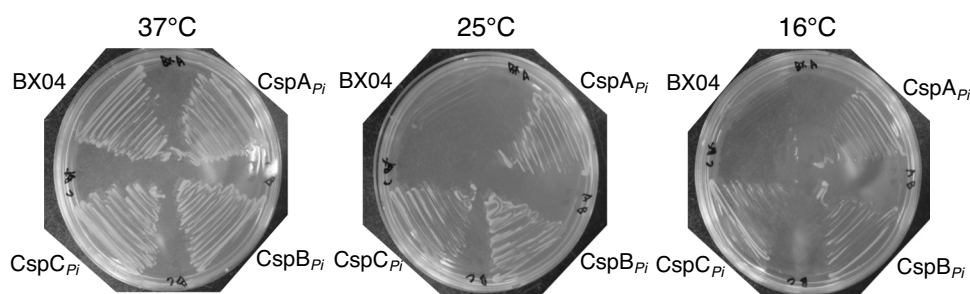


Fig. 4 – Overexpression of CspB_{PI} rescued cold-sensitive phenotype of csp quadruple-deletion *E. coli* strain, BX04. BX04 cells harboring pAED, pAED-cspA_{PI}, pAED-cspB_{PI}, or pAED-cspC_{PI} were grown in the liquid culture to an OD₆₀₀ of 0.4. The cells were then streaked on LB plates containing 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 CspB_{pi} greatly increased the cold tolerance of its recombinant host to an unprecedented level (Fig. 2). Overexpression of CspB_{pi} 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 CspB_{pi} would be of great academic interest and will be pursued in a future study. CspB_{pi} 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 CspB_{pi} 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, CspB_{pi} 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 *cspB*_{pi}-homologous genes, encoding both N-terminal extra domain and CSD, from evolutionarily related marine Flavobacteriaceae, including *Polaribacter* sp. MED152, *Lacinutrix* sp. 5H-3-7-4, *Winogradskyella* sp. PG-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 CspB_{pi} 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 CspB_{pi} 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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