RESEARCH LETTER

Structure and function of a cold shock domain fold protein, CspD, in *Janthinobacterium* sp. Ant5-2 from East Antarctica

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Abstract

A cold shock domain (CSD)-containing protein, CspD, of molecular mass ~7.28 kDa in a psychrotolerant Antarctic *Janthinobacterium* sp. Ant5-2 (ATCC BAA-2154) exhibited constitutive expression at 37, 22, 15, 4 and −1 °C. The *cspD* gene encoding the CspD protein of Ant5-2 was cloned, sequenced and analyzed. The deduced protein sequence was highly similar to the conserved domains of the cold shock proteins (Csps) from bacteria belonging to the class *Betaproteobacteria*. Its expression was both time- and growth phase-dependent and increased when exposed to 37 °C and UV radiation (UVC, dose: 1.8 and 2.8 mJ cm^{-2}). The results from the electrophoretic mobility shift and subcellular localization study confirmed its single-stranded DNA-binding property. *In silico* analysis of the deduced tertiary structure of CspD from Ant5-2 showed a highly stable domain-swapped dimer, forming two similar monomeric Csp folds. This study established an overall framework of the structure, function and phylogenetic analysis of CspD from an Antarctic *Janthinobacterium* sp. Ant5-2, which may facilitate and stimulate the study of CSD fold proteins in the class *Betaproteobacteria*.

Introduction

Microorganisms isolated from Antarctica are suitable candidates to study physiological and genetic mechanisms for the adaptation to cold and subzero temperatures. It is notable that a number of bacteria that have been isolated from the Antarctic subzero temperature environment are psychrotolerant, exhibiting optimum growth temperatures at or above 22 °C (Morita, 1975). Accumulation of proteins of the cold shock domain (CSD) family and the regulation of their corresponding genes is one of the adaptive responses to cold temperatures that has been described in both mesophilic and psychrotolerant bacteria including *Escherichia coli* (Phadtare *et al.*, 1999), *Bacillus subtilis* (Schindler *et al.*, 1999), *Arthrobacter globiformis* (Berger *et al.*, 1996), *Pseudomonas putida* (Gumley & Inniss, 1996), *Salmonella* spp. (Jeffreys *et al.*, 1998), *Rhodococcus* spp.(Bej *et al.*, 2000) and *Pseudomonas* sp.30-3 (Panicker *et al.*, 2002). The CSD has been reported to be an evolutionarily conserved nucleic acid-binding domain of ancient origin found in eubacteria. It is also homologous to the CSD in human Y-box protein YB-1 and to other eukaryotic Y-box proteins (Graumann & Marahiel, 1998).

The structures of cold-shock proteins (Csps) from different bacteria have been determined by either X-ray crystallography or nuclear magnetic resonance, for example *E. coli* CspA (Newkirk *et al.*, 1994; Schindelin *et al.*, 1994), *B. subtilis* CspB (Schnuchel *et al.*, 1993), *Bacillus caldolyticus* Csp (Mueller *et al.*, 2000), *Thermotoga maritima* Csp (Kremer *et al.*, 2001) and *Neisseria meningitidis* Csp (Ren *et al.*, 2008). All of them share a common OB (oligonucleotide/oligosaccharide-binding) fold consisting of five β-barrel sheets with two consensus RNA-binding domains (RNP1 and RNP2) placed side by side on separate β-sheets, comprising a high proportion of basic and aromatic residues. The binding of *B. subtilis* CspB and *B. caldolyticus* Csp with hexathymidine (dT6) involves stacking interactions between phenylalanine residues and the thymidine base, together with hydrogen bonds between the side chains of polar amino acids and pyrimidine bases (Max *et al.*, 2007).

Escherichia coli CspA family of proteins consist of nine homologs to the major cold-shock protein CspA (CS7.4) (Phadtare *et al.*, 1999) and they either function as a RNA chaperones by minimizing the secondary structure formation in mRNAs to allow efficient translation at low temperatures or as transcription regulators and transcription

antiterminators (Bae *et al.*, 2000). *Escherichia coli* CspA, CspB, CspG and CspI are cold inducible, whereas CspC and CspE are constitutively expressed and have been shown to function as suppressors of the temperature-sensitive *mukB106* mutation. The *mukB* gene is involved in the chromosome partitioning during cell division in *E. coli* (Yamanaka *et al.*, 1994). The expression of *E. coli cspF* and *cspH* has not been associated with any particular growth condition or phenotype (Giaquinto *et al.*, 2007). Non-cold-inducible *E. coli* CspD functions as a DNA replication inhibitor during the stationary growth phase. Its expression is inversely dependent upon the growth rate and induced upon glucose starvation at 37 °C (Yamanaka & Inouye, 1997). In another study, the function of the CspD in *Halobacterium* NRC-1 has been attributed to protection against UV damage of DNA, although the exact mechanism of such functions has not been elucidated (Baliga *et al.*, 2004). It has been shown that under antibiotic stress, *E. coli* CspD causes arrest in growth and reduced viability under the direct regulation of a motility and quorum-sensing regulator toxin–antitoxin pair, MqsR/MqsA, that possesses RNase activity and influence cell physiology (e.g. biofilm formation and motility) leading to the establishment of the persister population (Kim *et al.*, 2010; Kim & Wood, 2010).

It is apparent from the above findings that although all Csps contain highly conserved CSD, their physiological roles may vary depending upon the organisms, proteins and environment. Most structural and functional analyses were performed on Csps from mesophiles, but not from psychrophiles or psychrotolerant bacteria. Among all the classes of phylum *Proteobacteria*, the Csps from bacteria belonging to class *Betaproteobacteria* have not been characterized yet. Therefore, the major objective of this study was to elucidate the structure and physiological role of a cold-shock protein, CspD from a psychrotolerant *Janthinobacterium* sp. Ant5-2 isolated from Antarctica that has to survive extreme cold environment.

Materials and methods

Bacterial strains and growth media

Janthinobacterium sp. strain Ant5-2 (ATCC BAA-2154) was isolated from Lake Podprudnoye a small proglacial lake located in central Schirmacher Oasis, East Antarctica (latitude −70.766758°, longitude 11.610249°). Samples were collected aseptically in November 2008 from shallow water near the shoreline when ice-cover was absent and transported to the lab frozen and processed shortly after arrival. For all experiments in this study, Ant5-2 was grown in 1 : 2 (v/v) trypticase soy broth (TSB) (BD, Franklin, NJ). *Escherichia coli* BL21(DE3)pLysS (F - *omp*T *hsd*SB(rB -, mB -) *gal dcm* (DE3) pLysS (CamR) (Novagen, WI) was grown at 37 °C in Luria–Bertini (LB) medium.

Growth kinetics and freeze tolerance

For growth and cold tolerance, 250 mL of 1 : 2 (v/v) TSB medium was inoculated with 1 mL of overnight culture of Ant5-2 and incubated at 22 °C until the $OD_{450\,nm}$ reached 0.2. Then, 50 mL aliquots were transferred to incubator shakers set at 37, 22, 15, 4 and −1 °C. The OD was determined at various time intervals using a Lambda 2 spectrophotometer (Perkin Elmer, Norwalk, CT). To determine freeze tolerance, Ant5-2 culture was grown to exponential phase and frozen at −20 °C. The frozen culture was then subjected to one freeze–thaw cycle and viable plate count was determined before and after freezing as described previously (Panicker *et al.*, 2002).

Radiolabeling of total cellular proteins and immunoblot

Total cellular proteins of Ant 5-2 cultures were radiolabeled using EasyTag™ Express ^{35}S methionine Protein Labeling Mix (11 μCi μL^{-1}) (Perkin Elmer) and transferred immediately to −1, 4, 15 and 22 °C, respectively, and incubated for 1.5 h as described previously (Panicker *et al.*, 2002). The cell pellets were collected and proteins denatured at 98 °C for15 min in 2× sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel loading buffer (BioRad, Hercules, CA). The protein concentration was estimated colorimetrically using BCA Protein assay kit (Pierce, Rockford, IL); 50 μg proteins from each sample was separated in 12% (w/v) denaturing SDS polyacrylamide gel; the gel was dried and autoradiographed. For immunoblot assays, total proteins from Ant5-2 cultures incubated at −1, 4, 15, 22 and 37 °C and cultures exposed to different doses of UVC (290–100 nm) were extracted, transferred and immunoblotted with CapB antibody from *Pseudomonas* sp. 30/3 in duplicate as described previously (Panicker *et al.*, 2010). The membranes were scanned and the relative amount of CspD expressed in Ant5-2 was analyzed with IMAGEJ 1.40 software (http://rsb.info.nih.gov/ij/index.html).

Analysis of cold shock protein (Csp)

The cold shock gene was PCR amplified using primers designed from Csp of *Janthinobacterium lividum* (accession no. DQ074977), F-*cspD*-Ant5-2 (5′-dTTAGATTGGCTG AATGTTCGAAGCTTGC-3′) forward and R-*cspD*-Ant5-2 (5′-dATGGCAACTGGCATCGTAAAATGG-3′) reverse primers. The PCR amplification of the homologs of *E. coli cspA* on Ant5-2 genomic DNA was also attempted using a set of universal degenerate primers for *Enterobacteriaceae* CSPU5 (5′-dCCCGAATTCGGTAHAGTAAAATGGTTYAACKC-3′)

and CSPU3 (5′-dCCCGAATCCGGTTACGTTASCWGCTK SHGGDCC-3′) (Francis & Stewart, 1997). The cycling parameters, cloning and sequencing method used was as described previously (Panicker *et al.*, 2010). The deduced amino acid sequence of CspD from Ant5-2 was aligned with homologous sequences obtained from BLAST P and with protein sequences in NCBI database using CLUSTAL X (http://www.clustal.org/) and the phylogenetic trees were generated by neighbor joining method and MEGA version 4.0 (http://www.megasoftware.net/) software (Tamura *et al.*, 2007).

In situ localization of Csp (Ant5-2) by immunofluorescence staining

Ant5-2 culture was grown in 1 : 10 (v/v) TSB growth medium at 22 °C until $OD_{450\,nm} = 0.3$; then aliquots of 50 mL were exposed to 4, 15 and 22 °C, respectively, for 1 h. The fixation, permeabilization and staining of cells with rabbit anti-CapB antiserum (1 : 1500 dilutions in bovine serum albumin–phosphate-buffered saline (PBS), followed by HiLyte Fluor 488-labeled goat anti-rabbit IgG secondary antibody (AnaSpec, San Jose, CA) along with 4′-6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO) was performed as described previously (Panicker *et al.*, 2010). Slides were mounted in PBS-glycerol (pH 7.2) solution and observed under a Leica™ fluorescence microscope (Bannockburn, IL).

Electrophoretic mobility shift assay (EMSA)

cspD was PCR amplified using forward F-BamH1-CspD-Ant5-2 (5′-dGCCAGGATCCATGGCAACTGGCATC-3′) and reverse R-XhoI-CspD-Ant5-2 (5′-dGCCACTCGAGT TAGATTGGCTGAATG-3′) primers from Ant 5-2 cells and cloned into pET45b(+) plasmid (Novagen, WI). The CspD from Ant5-2 protein was expressed in *E. coli* BL21 (DE3) by inducing with 1 mM isopropyl-β-D-thiogalactopyranoside for 6 h and then purified using the His.Tag kit (Novagen). DNA-binding assay was performed by incubating purified CspD of Ant5-2 with 0.72 ng of 34-mer randomized oligonucleotide sequence (5′-dGCCAGAATTCTTAGATTGGCT GAATGTTCGAAGC-3′) and double stranded (ds)-DNA (PCR product) using fluorescence-based EMSA kit (Molecular Probes) according to the manufacturer's instructions.

Homology modeling and docking

The primary and secondary structure analyses were performed using the PROTPARAM tool on ExPASy server (Bairoch *et al.*, 2005) and PSIPRED (Jones, 1999), respectively. The tertiary structure of CspD from Ant5-2 was generated by the MODELLER software from HHPRED alignments on HHpred servers (Soding *et al.*, 2005; Eswar *et al.*, 2006). The significance of the protein structure similarity was measured by TM-score calculated by T-align, a more sensitive method than root-mean-square-deviation (Zhang & Skolnick, 2005). The protein–protein docking was performed using the HEX 5.1 software according to its manual (Ritchie & Venkatraman, 2010).

Results

Growth kinetics and freeze tolerance of Ant5-2

During the initial 4 h, Ant5-2 cultures exhibited faster growth rates at 22 and 37 °C than at 15 °C (Fig. 1). Within 20 h of incubation at 15, 22 and 37 °C, the cultures grew exponentially and reached stationary phase. However, the culture at 37 °C exhibited a decline in growth after 48 h of incubation. In contrast, the cultures maintained at −1 and 4 °C did not show any significant cell proliferation for 24 and 4 h, respectively. Thereafter, the culture at 4 °C exhibited exponential growth and reached stationary phase within 72 h. A slow but steady exponential growth of the culture at −1 °C was noticed after incubation for 24 h. After one freeze–thaw cycle, increased survival was observed when Ant5-2 was exposed to 4 °C before freezing. The cultures transferred to 4 °C showed 94.1% survival compared with the 48.9% survival of cultures incubated at 22 °C (Supporting Information, Fig. S1).

Identification and expression of CspD in Ant5-2

The autoradiogram exhibited expression of a ~7.28-kDa Csp (CspD) at all temperatures (Fig. S2). The immunoblot results showed that the expression of CspD in Ant5-2 was both time- and growth phase-dependent (Fig. 2a). Its

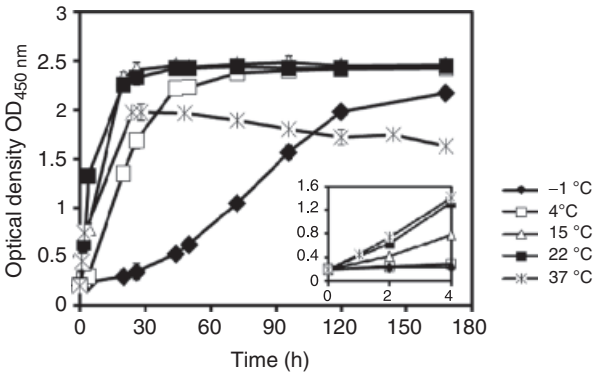

Fig. 1. Spectrophotometric analysis of the growth of *Janthinobacterium* sp. Ant5-2 (ATCC BAA-2154) in 1 : 2 (v/v) TSB medium at various temperatures during 0 and 160 h of growth measured at $OD_{450\,nm}$. Ant5-2 culture was maintained at 22 °C till $OD_{450\,nm}$ reached 0.2 and then transferred and grown at 37, 22, 15, 4 and −1 °C, respectively. Comparable results were obtained from two separate studies. Growth curve of the same cultures between 0 and 4 h also measured at $OD_{450\,nm}$ is shown in the inset.

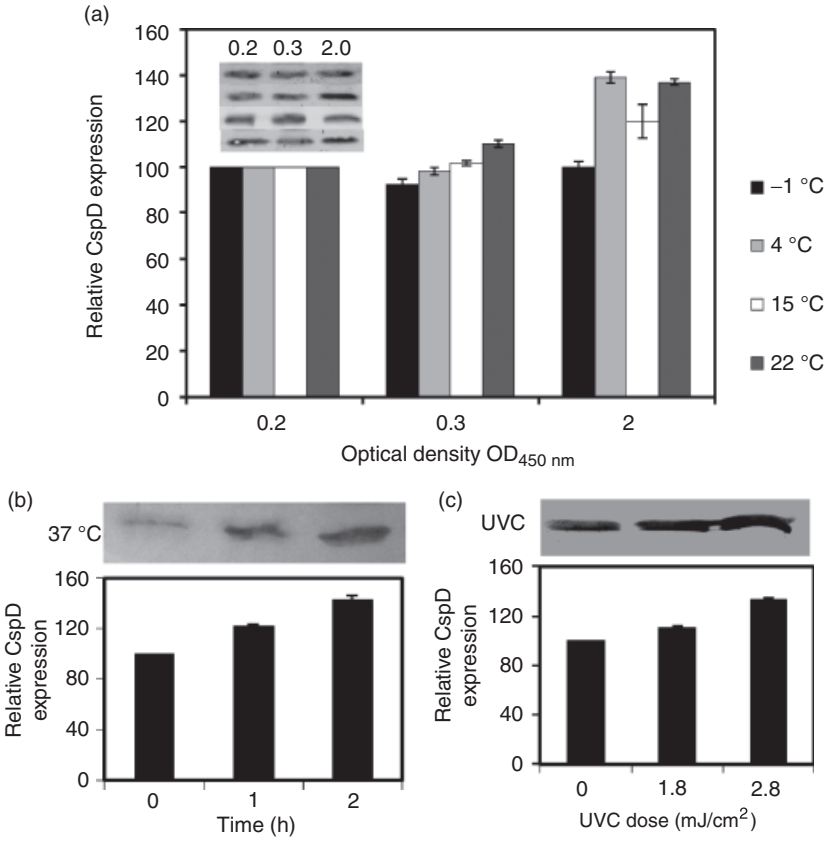

Fig. 2. (a) Immunoblot analysis of the expression of CspD protein in Ant5-2 following exposure to various temperatures at different phases of growth are represented as bar graphs with representative blots in the inset. The intensity of the bands from culture exposed to 22 °C and $OD_{450\,nm} = 0.2$ was used as 100% and compared to determine the relative intensities of the cultures treated at various temperatures. Each data point is an average of two blots from identical experiments treated similarly and the error bar represents SE. Similarly, the expression analyses were performed at a higher growth temperature of 37 °C (b) and UV exposure (c) and represented as bar graphs with representative immunoblots.

expression increased at 37 °C and UVC exposure (Fig. 2b and c).

A 204-bp DNA fragment encompassing the entire ORF of the *cspD* gene (accession no. HQ873479) was PCR amplified. The deduced amino acid sequence exhibited 100% identity with the cold shock transcription regulator of *J. lividum* DSM 1522 and > 98% sequence identity with the RNA chaperone, transcription antiterminator of *Herminiimonas arsenicoxydans* and with Csps from different bacteria belonging to class *Betaproteobacteria* (Figs 3 and Fig. S3). The CspD from Ant5-2 showed highest identity and similarity to *E. coli* CspE (67/83%) and *E. coli* CspD (56/74%) when compared with Csps from *E. coli*, *B. subtilis* and *Pseudomonas* sp. 30/3 (Fig. 3). PCR amplification of the *cspA* family of genes in Ant5-2 genomic DNA using CSPU5 and CSPU3 universal primers (Francis and Stewart, 1997) resulted in negative outcome (Fig. S4).

Phylogenetic analysis of CspD from Ant5-2

The phylogenetic analysis revealed that the Csps from bacteria belonging to the class *Betaproteobacteria* including CspD from Ant5-2 group together in a single cluster with low bootstrap value, i.e. 40% (Fig. 3). Csps from *E. coli* and *B. subtilis* also grouped separately with bootstrap values of 50% and 42%, respectively, with the exception of *E. coli* CspD, which aligned more closely to the *Betaproteobacteria* node with a low bootstrap value of 37%. DEAD-box RNA helicase containing CSD from Archaea *Methanococcoides burtonii* (AAF89099) was used as an outgroup,

Subcellular localization and DNA-binding activity of CspD in Ant5-2

Immunofluorescence staining was used to localize CspD using the anti-CapB rabbit-antiserum at different temperatures to determine the possible cellular role of CspD in Ant5-2. The cellular location of the nucleoid was confirmed by DAPI staining (Fig. 4a, c, and e). At 4 °C, a dense accumulation of the anti-CapB antibody immunoconjugated with the green Hilyte Fluor 488-labeled goat anti-rabbit IgG secondary antibody was observed in and around the nucleoid region (Fig. 4a and b). At 15 and 22 °C, the green fluorescence was dispersed in the cytosol as well as in the nucleoid region (Fig. 4c–f). The purified CspD protein from Ant5-2 (Fig. S5) exhibited binding affinity with single stranded (ss)-oligonucleotides with increasing

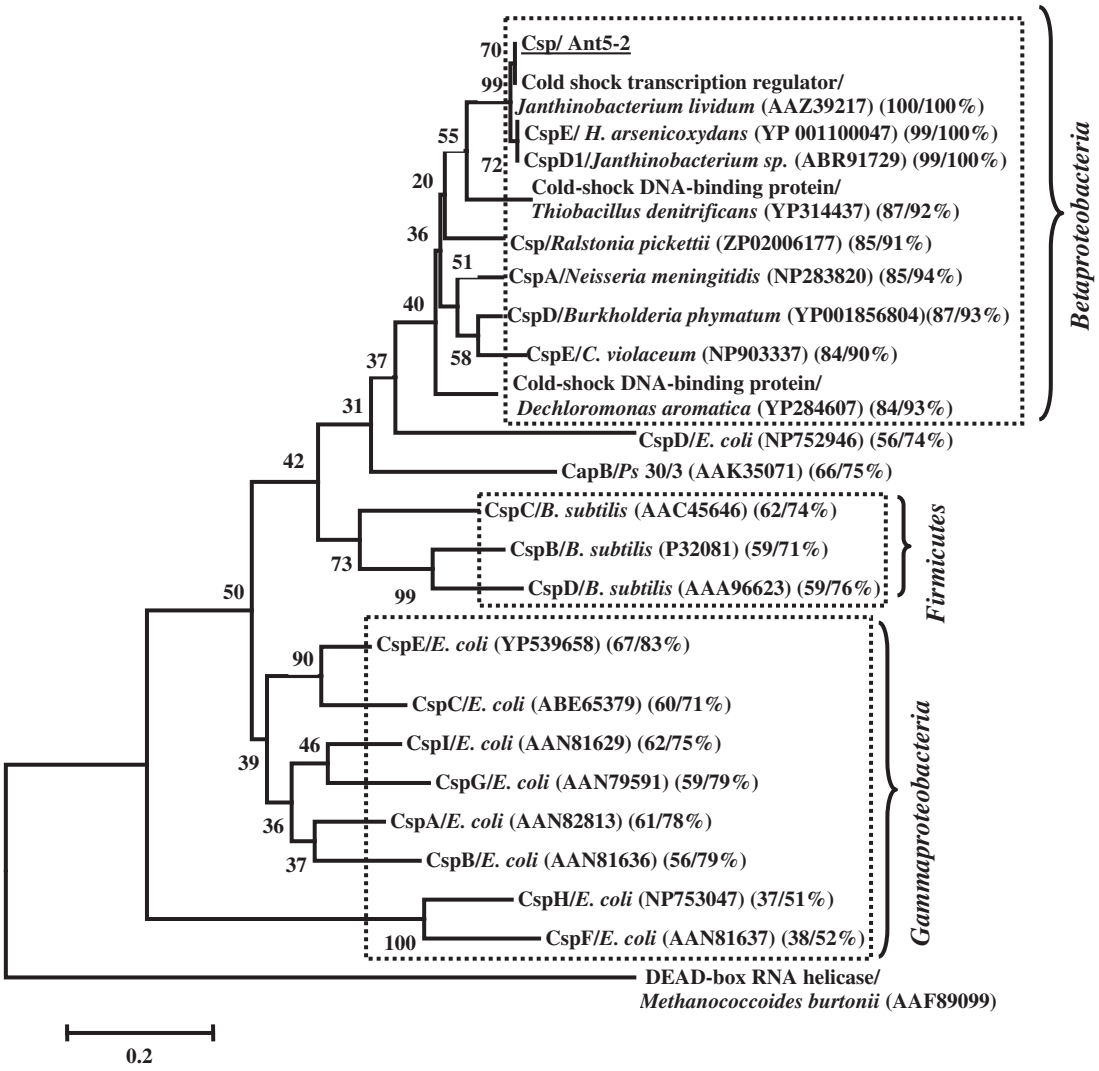

Fig. 3. Evolutionary relationships of the CspD from Ant5-2 with Csps from other bacterial species inferred using the neighbor-joining method. The percentage of replicate trees in which the associated bacterial species clustered together in the bootstrap test (1000 replicates) is shown next to the branches. The accession numbers and the percent amino acid sequence identities/similarities of Csps with CspD from Ant5-2 are shown in parentheses.

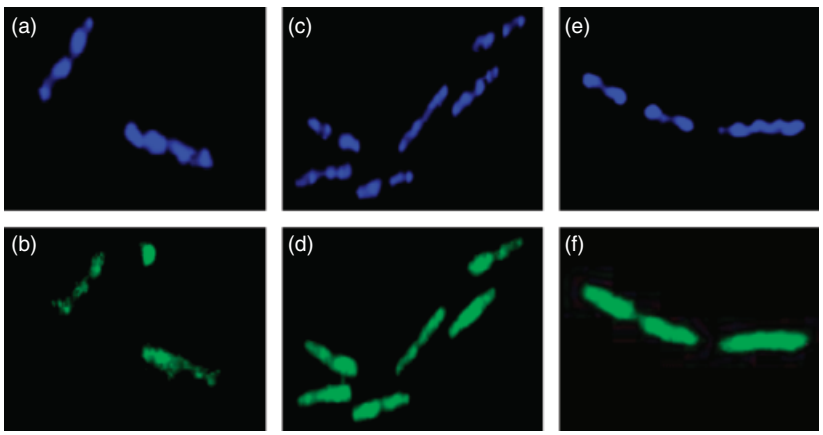

Fig. 4. *In situ* immunolocalization of CspD in Ant5-2 at various temperatures; (a, c, e) The DAPI-stained (blue fluorescence) nucleoids at 4, 15 and 22 °C, respectively; (b, d, f) The CspD-bound HiLyte Fluor 488 (green fluorescence) at 4, 15 and 22 °C, respectively. CspD can be seen localized around the compact nucleoid region at 4 °C (b) and CspD localized both around nucleoid and cytosol at 15 and 22 °C (d, f).

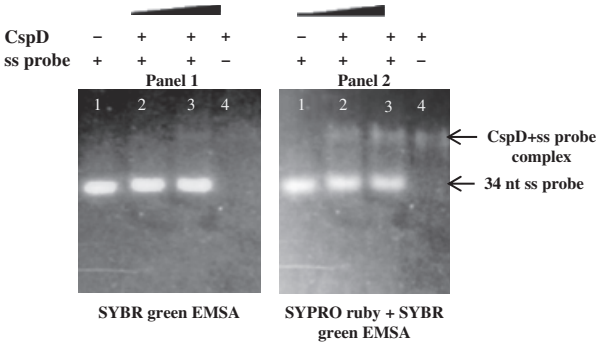

Fig. 5. The polyacrylamide gel showing the DNA-binding activity of CspD with single-stranded oligonucleotide (5′-GCCAGAATTCTTA-GATTGGCTGAATGTTCGAAGC-3′). Panel 1: gel was stained with SYBR® Green EMSA to visualize oligonucleotide DNA probe either complexed with or without the CspD protein. Lane 1, 0.72 ng ss-oligonucleotide probe only; lanes 2 and 3, 0.72 ng ss-oligonucleotide probe complexed with 2 and 3 μg of CspD protein, respectively; lane 4, CspD protein only. Panel 2: the same gel in panel 1 was stained with SYPRO Ruby EMSA stain to visualize CspD protein and merged image of both stains is shown. The lanes in the panel 2 gel correspond to the lanes described in panel 1. Note that the high-molecular-weight bands in lanes 2 and 3 represent the CspD complexed with the ss-oligonucleotides. +, the ss-oligonucleotide or the CspD added in the reaction; −, the ssDNA or the CspD protein was not added in the reaction; '▬' represents the concentration gradient of the CspD protein in the reaction.

concentration (Fig. 5) and not with dsDNA (PCR product) (data not shown).

Molecular structure of CspD from Ant5-2 *in silico*

Based on the amino acid residues and use of the homology modeling approach, the secondary and the tertiary structures of CspD from Ant5-2 indicated that the aromatic residues are conserved and three of the eight aromatic residues were docked on the nucleic acid-binding surface, F15 (F12), F17 (F20), and F28 (F31) (amino acid numbering on *E. coli* CspA is indicated in parentheses) (Feng *et al.*, 1998). CspD from Ant5-2 has five basic and three acidic residues on the nucleic acid-binding surface. Its calculated theoretical isoelectric point (pI) was 5.6. Five β-strands and one α-helix were identified by the secondary-structure prediction (Fig. 6a). The solvent-exposed basic amino acids were K7 in β1 strand, K13 in L1, H30 in β3, K40 in L3 and K57 in L4 located on the nucleic acid-binding surface (Fig. 6b). The tertiary structure was designed with *N. meningitidis* CSD protein (*Nm*-Csp) (PDB reference: 3CAM) using the template provided by HHPRED and MODELLER software (Soding *et al.*, 2005; Eswar *et al.*, 2006). The structure of the monomer of CspD from Ant5-2 consists of two subdomains of similar length separated by a long loop. Subdomain 1 includes β-strands 1–3 and subdomain 2 contains a β-ladder comprising strands 4 and 5 (Fig. 6a and b). The TM-score of the predicted structure was calculated to be 0.96738. It has been reported that the *Nm*-Csp form a dimer in the crystallographic asymmetric unit consisting of two five-stranded β-barrels (Ren *et al.*, 2008). Because protein pairs with a TM-score > 0.5 are mostly in the same fold (Xu & Zhang, 2010), we tested whether CspDAnt5-2 form a dimer-like *Nm*-Csp by docking monomer pairs with the HEX 5.1 software (Ritchie & Venkatraman, 2010). The software predicted that the two modeled CspD monomers from Ant5-2 form a highly stable dimer like *Nm*-Csp with E-total -1278.08 and E-shape: -1107.25, the lowest interaction free energy and the site of interaction, respectively (Fig. 6c and d).

Discussion

This study encompasses structural, functional and molecular phylogeny-based evolutionary analysis of the cold shock protein, CspD, from an Antarctic bacterium *Janthinobacterium* sp. Ant5-2 belonging to the CSD family of proteins in *Betaproteobacteria*. *Janthinobacterium* sp. Ant5-2 is a psychrotolerant bacterium, which can tolerate a wide range of temperature ranging between 37 and $-1\,^{\circ}C$ as evident from the growth curve (Fig. 1). The slow growth rate at $-1\,^{\circ}C$ could be possibly due to the decreased cellular metabolic activities at cold temperatures. It was found that Ant5-2 culture exposed to an intermediate temperature of $4\,^{\circ}C$ have a survival advantage upon freezing followed by thawing compared with the untreated cultures. In this study, the *cspD* gene sequence in Ant5-2 was identified by PCR amplification using oligonucleotide primers based on a similar gene sequences from its closest relative *J. lividum* (accession no. DQ074977). The complete genome sequences of yet another close relative of Ant5-2, i.e. *Janthinobacterium* sp. *Marseille* (accession no. NC009659) consist of only two copies of *cspD*, i.e. *cspD1* (accession no. YP001353208) and *cspD2* (accession no. YP001354206), and the fosmid library sequences of *J. lividum* (accession no. DQ074977) consist of one *csp* (accession no. AAZ39217). No other sequences similar to the other Csp proteins have been identified on the genome of these *Janthinobacterium* spp. Furthermore, we have shown that PCR amplification of the Ant5-2 genomic DNA using the universal oligonucleotide primers CSPU5 and CSPU3 targeting the *csp* family of genes resulted in negative result (Fig. S3). This suggests that CspD is most likely the only protein from CSD family of proteins present in Ant5-2.

The cold-shock responses in mesophilic and psychrophilic bacteria are found to be different, including the lack of repression of housekeeping protein synthesis and the presence of cold-acclimation proteins in psychrophiles (D'Amico *et al.*, 2006). Many of the Csp family of proteins in mesophiles are constitutively expressed and function as cold

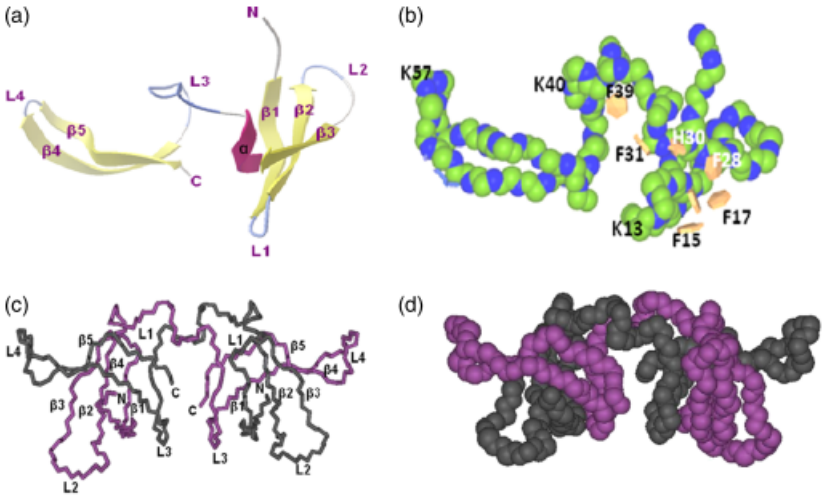

Fig. 6. (a) Three-dimensional structure model of CspD monomer from Ant5-2 predicted by HHPRED alignment and MODELLER showing the secondary structures as ribbon diagram with all the five β-strands and four loops labeled from N- to C-terminal. (b) 3D structure of CspD showing the solvent-exposed aromatic (indicated by aromatic rings) and basic amino acids. (c) Ribbon diagram showing the CspD dimer from Ant5-2 as predicted by HEX 5.1 software; the two polypeptide chains are colored grey and magenta and secondary-structure features are numbered. (d) Predicted CspD dimer structure from Ant5-2 with swapped domains in ball and stick drawing.

acclimation proteins in psychrophiles (D'Amico *et al.*, 2006). Our results demonstrate that unlike *E. coli* and like psychrophiles, the CspD in Ant5-2 is constitutively expressed at cold temperatures (Fig. 2a). Its subcellular localization in and around the nucleoid region at 4 °C and its binding affinity with ss-oligonucleotide probes that possessed randomized sequences indicated that CspD in Ant5-2 may bind through hydrophobic interaction to ssDNA without apparent sequence specificity (Figs 4 and 5), further supporting its possible role as a RNA chaperone at cold temperatures. In a previous complementation study, the Csp and CSD fold proteins of Archaea was able to function effectively to rescue a growth defect in a cold-sensitive *E. coli* strain, BX04 (Giaquinto *et al.*, 2007). It illustrates that Csps and CSD fold proteins have retained a high degree of functional similarity. In addition we observed that CspD expression in Ant5-2 increased at 37 °C and upon UV exposure (Fig. 2b and c), and as described previously (Yamanaka *et al.*, 2001; Kim & Wood, 2010), the cells also become elongated at 37 °C (data not shown), indicating that the CspD in Ant5-2 is a stress-inducible protein. Because CspD in Ant5-2 shares structural similarity with *E. coli* CspD, it might retain the same function as DNA replication inhibitor at 37 °C. It has also been reported that PprM, a homolog of Csp and a homodimer like *E. coli* CspD, is involved in the expression of many protein(s) that are important for the radioresistance of *Deinococcus radiodurans* (Ohba *et al.*, 2009).

In this study, we have shown that the overall fold of the predicted CspD monomer from Ant5-2 did not closely resemble those of other bacterial cold-shock proteins. In both *E. coli* CspA and *Bs*-CspB, each chain is folded into an independent three-dimensional biological unit whereas the predicted Ant5-2 CspD dimer is composed of the N-terminal residues 1–36 from one chain and the C-terminal residues 37–67 from the other chain. The stable dimer prediction was performed with the help of the HEX 5.1 docking software, which is considered to be a more reliable platform for 'protein–protein' compared with 'protein–ligand' docking. The predicted CspD dimer from Ant5-2 was formed by the exchange of two β-strands between protein monomers, but formed a symmetric unit of 2 five-stranded β-barrels unlike *Nm*-Csp that form two asymmetric five-stranded β-barrels. Despite differences, the predicted CspD dimer in Ant5-2 had significant structural similarities with the *Nm*-Csp and *Bs*-CspB dimers (Ren *et al.*, 2008), sharing the same folds as that of monomeric Csps. This implies that it binds to ssDNA in a similar fashion. As evident from the electrostatic properties, the only DNA-binding region in the predicted tertiary structure of CspD dimer of Ant5-2 is the side of the β-barrel, which corresponds to the DNA-binding site in OB-fold proteins such as *E. coli* CspA and *B. subtilis* CspB. Although its theoretical pI is 5.6, the attractive potential for nucleic acids is created by the solvent-exposed basic amino acids located on the nucleic acid-binding surface. The solvent-exposed aromatic residues on the surface of these molecules also bind and melt nucleic acid secondary structure to facilitate transcription and translation at low temperatures (Phadtare *et al.*, 2004).

The phylogenetic relationship of the CspD from Ant5-2 and Csps from three classes of phylum *Proteobacteria*, i.e. *Betaproteobacteria*, *Gammaproteobacteria* and *Firmicutes*, revealed that they distinctly form orthologous protein groups indicating a speciation event at each node except *E. coli* CspD. It should be noted that the low bootstrap values obtained in this study might be due to the highly conserved nature of CSD among different bacteria and also different Domains of life and obtaining high bootstrap values may require a relatively large amount of data (Berbee

et al., 2000). Unlike *Nm*-Csp, CspD from *Janthinobacterium* sp. Ant5-2 is the only representative Csp from class *Betaproteobacteria* whose structure and function analyses illustrate its role in cold adaptation. Because *N. meningitidis* are commensal organisms that reside in the human upper respiratory tract, the role of *Nm*-Csp could not be described in context of cold adaptation (Ren *et al*., 2008).

In conclusion, we have described the growth characteristics, expression and overall structure and function of CspD in a psychrotolerant Antarctic bacterium *Janthinobacterium* sp. Ant5-2. Our principal finding is that CspD appears to undergo domain swapping to form stable dimeric structures and possess ssDNA-binding activity essential for cold and UV adaptations in extreme Antarctic environment.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Viable cell count of Ant5-2 after a single freeze–thaw cycle.

Fig. S2. Autoradiogram of ^{35}S-methionine-labeled total cellular proteins from Ant5-2 cultures at different temperatures.

Fig. S3. Multiple sequence alignment of deduced amino acid sequence of the cold shock protein CspD from Ant5-2 with the cold shock transcriptional regulator sequence from *J. lividum*, CspE from *H. arsenicoxydans*, CspD1 from *Janthinobacterium* sp. Marseille, cold-shock DNA-binding protein family protein from *T. denitrificans* ATCC 25259, cold-shock DNA-binding protein family protein from *D. aromatica* RCB, CspD from *B. phymatum* STM815, CspA from *N. meningitidis* Z2491, cold shock protein from *R. pickettii* 12D and CspE from *C. violaceum* ATCC 12472.

Fig. S4. The agarose gel (1% w/v) showing the results of PCR amplification with CSPU5 and CSPU3 universal primers CSPU5 and CSPU3.

Fig. S5. SDS-polyacrylamide gel (12%, w/v) electrophoresis showing purified CspD of Ant5-2 expressed in *Escherichia coli*.

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