The Protein Journal, Vol. 26, No. 1, January 2007 (© 2006)
DOI: 10.1007/s10930-006-9044-1
Published online: January 3, 2007

Characterization of Cold-Shock Protein A of Antarctic *Streptomyces* sp. AA8321

Min-Jung Kim,[1] Yoo Kyung Lee,[2] Hong Kum Lee,[2] and Hana Im[1,3]

Polar organisms should have mechanisms to survive the extremely cold environment. Four genes encoding cold-shock proteins, which are small, cold-induced bacterial proteins, have been cloned from the Antarctic bacterium *Streptomyces* sp. AA8321. Since the specific functions of any polar bacterial or *Streptomyces* cold-shock proteins have not yet been determined, we examined the role of cold-shock protein A from *Streptomyces* sp. AA8321 ($CspA_{St}$). Gel filtration chromatography showed that purified $CspA_{St}$ exists as a homodimer under physiological conditions, and gel shift assays showed that it binds to single-stranded, but not double-stranded, DNA. Overexpression of $CspA_{St}$ in *Escherichia coli* severely impaired the ability of the host cells to form colonies, and the cells developed an elongated morphology. Incorporation of a deoxynucleoside analogue, 5-bromo-2′-deoxyuridine, into newly synthesized DNA was also drastically diminished in $CspA_{St}$-overexpressing cells. These results suggest that $CspA_{St}$ play a role in inhibition of DNA replication during cold-adaptation.

KEY WORDS: Cell elongation; cold-shock protein; DNA binding protein; *Streptomyces*; replication inhibitor.

1. INTRODUCTION

Temperature downshift is one of the major environmental challenges facing bacteria, especially at the North and South Poles. At low temperatures, decreased membrane fluidity hinders crucial membrane functions such as transport and protein movement. In addition, formation of stable secondary structures in DNA and RNA may inhibit transcription and translation, and association of an inhibitory factor with ribosomes may reduce protein translation (Phadtare, 2004). To cope with these challenges, exponentially growing cells express a number of cold-induced proteins upon temperature drops of more than 10°C (Jones *et al.*, 1987). Of the cold-induced proteins, the small proteins known as cold-shock proteins (Csps) are the most prominent.

Csps are widely found in various bacteria, including psychrophiles, mesophiles, and even thermophiles (Ermolenko and Makhatadze, 2002; Phadtare *et al.*, 2003). Most bacteria appear to have several *csp* genes. For example, *Escherichia coli* has nine *csp* homologues (*cspA–cspI*; Yamanaka *et al.*, 1998), and *Bacillus subtilis* has three (*cspB, cspC, and cspD*; Graumann *et al.*, 1996). Several Csps have been reported in *Streptomyces* spp., which are soil microorganisms that are

[1] Department of Molecular Biology, Sejong University, 98 Gunja-dong, Kwangjin-gu, Seoul, 143-747, Korea.
[2] National Research Lab for Marine Microbial Diversity, Polar BioCenter, Korea Polar Research Institute, Korea Ocean Research and Development Institute, 406 Getbul Tower, Songdo Technopark, Incheon, 406-840, Korea.
[3] To whom correspondence should be addressed. e-mail: hanaim@sejong.ac.kr

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; CD, circular dichroism; Csp, cold shock protein; $CspA_{St}$, cold-shock protein A from *Streptomyces* sp. AA8321; dNTP, deoxynucleotide triphosphate; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA.

exposed to various physical and chemical stresses, including heat-shock and cold-shock. A Csp-like protein was inadvertently identified in *Streptomyces clavuligerus* by amino acid sequencing during purification of thioredoxin (Av-Gay *et al*., 1992). More recently, five proteins that cross-react with antibodies raised against *B. subtilis* Csps were identified in *Streptomyces aureofaciens* (Mikulík *et al*., 1999). Genome sequencing of the industrially important microorganisms *Streptomyces* spp. identified three *csp*-like genes in each of *Streptomyces avermitilis* MA-4680 (Omura *et al*., 2001; Ikeda *et al*., 2003) and *Streptomyces coelicolor* A3(2) (Bentley *et al*., 2002).

However, not all *csp*-like genes are induced by cold shock, and not all appear to be essential. Of the nine *E. coli* Csps, only four (CspA, CspB, CspG, and CspI) are induced by temperature downshift (Nakashima *et al*., 1996; Wang *et al*., 1999). Simultaneous deletion of two or three cold-inducible *csp* genes does not affect cell viability at low temperatures. In fact, some Csps may have redundant functions, as shown by an *E. coli* strain with triple *csp* deletions (Δc*spA*, Δ*cspB*, Δ*cspG*). This strain exhibits increased expression of CspE, probably as a compensatory mechanism. Sensitivity to temperature downshift in this strain occurs only upon addition of a fourth deletion, Δ*cspE* (Xia *et al*., 2001). In cases of *Streptomyces* Csps, two (CspB and CspC) of five *S. aureofaciens* Csp proteins are induced by cold-shock (Mikulík *et al*., 1999). Like CspC and CspE of *E. coli*, Csp1 of the antibiotic-producing bacterium *Streptomyces hygroscopicus* is expressed constitutively and is not induced by cold-shock (Martínez-Costa *et al*., 2003). Although CspA expression in *S. coelicolor* A3(2) increases dramatically upon temperature down-shift, disruption of *cspA* has no effect on growth or morphological differentiation either at optimal or low temperatures (Kormanec and Ševčíková, 2000), possibly due to the redundant Csps.

Csps have molecular masses of about 7 kDa and are about 70 amino acid residues in length. The known three-dimensional structures of Csps, including *E. coli* CspA (Schindelin *et al*., 1994), *B. subtilis* CspB (Schindelin *et al*., 1993), and *Thermotoga maritima* CspB (Kremer *et al*., 2001) are very similar. Each is composed of five antiparallel β-strands that comprise a β-barrel structure known as a cold-shock domain-fold. This fold contains the canonical amino acid sequences known as RNA binding motifs, RNP1 and RNP2 (Schindelin *et al*., 1993; Landsman, 1992), which are implicated in binding to single-stranded DNA (ssDNA) and RNA (Jiang *et al*., 1997; Lopez and Makhatadze, 2000).

The best understood Csps are the CspA family of *E. coli*. CspA of *E. coli* may function as an RNA chaperone by destabilizing RNA secondary structure (Jiang *et al*., 1997), thus allowing efficient translation and modulating transcription. On the other hand, expression of CspD is induced in *E. coli* during stationary phase or upon nutrient depletion (Yamanaka and Inouye, 1997), and it has been suggested that CspD may function as a DNA replication inhibitor (Yamanaka *et al*., 2001). The cold-sensitive phenotype of the *E. coli* quadruple *csp* deletion strain described above (Δc*spA*, Δ*cspB*, Δ*cspG*, Δ*cspE*) is suppressed by overexpression of any of the Csp homologues except CspD (Xia *et al*., 2001). The result suggests a unique, non-redundant role for CspD, despite of its high sequence homology with other Csps.

Although Csp sequences are highly conserved, their physiological roles may vary depending on the proteins and organisms. Most functional analyses were done on Csps from mesophiles and thermophiles, but not from psychrophiles, which have to survive the extremely cold environment. Recently, we have cloned four *csp*-homologous genes from the Antarctic *Streptomyces* sp. AA8321. Since the specific functions of any polar bacterial or *Streptomyces* Csp proteins have not yet been determined, we chose to characterize $CspA_{St}$ from the Antarctic bacterium *Streptomyces* sp. AA8321.

2. MATERIALS AND METHODS

2.1. Chemicals

Hiload Superdex™ 75 16/60 column, Q-sepharose™ fast flow resins, lysozyme, and Hybond™ ECL™ nitrocellulose membrane were from Amersham Biosciences Co. (Piscataway, NJ, USA). Streptavidin-peroxidase and bovine serum albumin were purchased from Sigma (St. Louis, Missouri). 5′-Biotinylated oligonucleotides were manufactured by Bioneer Co. (Daejeon, Korea). Centricon was purchased from Amicon Inc. (Beverly, MA, USA), and isopropyl-β-D-1-thiogalactoside (IPTG) was from Duchefa (Haarlem, Netherland). Curix CP-BU, a medical X-ray film,

was purchased from Agfa Co. (Ridgefield Park, NJ, USA), and Bio-Rad DC (detergent compatible) protein assay kit was purchased from Bio-Rad Laboratories Inc. (Hercules, CA, USA). Cellular DNA fragmentation ELISA kit was purchased from Roche Applied Science (Basel, Switzerland). All other chemicals were reagent grade.

2.2. Cloning, Expression, and Purification of $CspA_{St}$

To overproduce $CspA_{St}$ in *E. coli*, pBAD-TOPO DNA containing *cspA* gene from *Streptomyces* sp. AA8321 was double-digested with *Nde*I and *Bam*HI restriction endonucleases, and DNA fragment of 0.2 kb in size was then transferred to pAED4 vector (Doering, 1992) digested with the same enzymes. The resulting plasmid for $CspA_{St}$-expression in *E. coli* was named as pAED-cspA$_{St}$. A previously described pAED4-derived plasmid for expression of recombinant human α_1-antitrypsin, pFEAT30 (Im *et al.*, 2002), was used as a control in cell growth experiments.

The recombinant plasmids were transformed into competent *E. coli* BL21(DE3) pLysS cells (Invitrogen Co., Carlsbad, California, USA). Transformed cells were grown on Luria–Bertani (LB) medium plates supplemented with 100 μg/ml ampicillin and 30 μg/ml chloramphenicol (USB Co., Piscataway, NJ, USA). For overproduction of $CspA_{St}$, 1 ml of the overnight liquid culture was transferred to a flask containing 50 ml fresh LB medium containing the same antibiotics. Cells were grown with vigorous shaking to a density of $OD_{600} \approx 0.2$, and overproduction of recombinant protein was induced by addition of IPTG to final concentration of 0.1 mM. The cultures were incubated further at 37°C for 3 h with vigorous shaking.

For purification of $CspA_{St}$, cells from 1 L culture were harvested by centrifugation, and resuspended in 40 ml of 20 mM Tris–HCl buffer, pH 8.0. Cells were lyzed by sonication using a Bandelin Sonoplus HD2200 ultrasonic homogenizer (Berlin, Germany) with a TT13 tip at 50% cycle (1 min × 3 times), and the supernatant and pellet fractions were separated by centrifugation (Hanil Supra22 K, Inchun, Korea) at 10,000 rpm at 4°C for 30 min. The supernatant fraction was dialyzed against 20 mM Tris–HCl buffer, pH 8.0, and loaded on a Q-sepharose^{TM} fast flow ion exchange column equilibrated with the same buffer. $CspA_{St}$ protein was eluted with a $0 \sim 0.5$ M NaCl gradient, and $CspA_{St}$-containing fractions were pooled and size-fractionated through a Centricon with a molecular cut-off of 30 kDa. Filtrates were collected, and the purity of proteins was analyzed by 20% SDS-PAGE. The protein bands were visualized by Coomassie brilliant blue R250 staining. Concentrations of proteins were determined using Bio-Rad DC (detergent compatible) protein assay kit.

2.3. Circular Dichroism (CD) Measurement

CD spectra were collected using a Jasco-720 spectropolarimeter in a 1 mm path-length cell. Temperature was controlled at 15°C by a Jasco PTC343 connected to a water bath. Concentration of $CspA_{St}$ was 17 μM in 20 mM Tris–HCl, pH 8.0. Scans were repeated for seven times for each sample at a scan speed of 20 nm per min.

2.4. Microscopic Observation of $CspA_{St}$-overproducing Cells

At 3 h after IPTG addition, *E. coli* cells containing various plasmids were fixed on a glass slide, and stained with 2.5% Safranin. Cell morphology was observed using a CH20BIMF200 microscope (Olympus Optical Co., Japan).

2.5. DNA Binding Assays

5′-Biotynylated primers of sequence 5′-CCATGGGCAGCAGCCATCAT-3′ (primer 1) and 5′-TCGAGTGCGGCCGCAAGCTT-3′ (primer 2) were synthesized by Bioneer Co. (Daejeon, Korea). A 143 bp *Nde*I-*Xho*I fragment of pET28a (Novagen, Darmstadt, Germany) was amplified by PCR, using primer 1 and primer 2. PCR products were purified on 2% agarose gels. When the single-stranded DNA was used as a probe, the PCR products were heat-denatured and transferred to ice immediately.

The standard binding assay contains 50 ng of DNA and 2 μg of purified $CspA_{St}$. The reaction buffer was 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 50 mM KCl, and 7.4% glycerol. The reaction was carried out on ice for 20 min in a 20 μl final reaction volume. The reaction products were separated on an 8% acrylamide gel, using TBE (50 mM Tris–boric acid, 1 mM EDTA, pH 8.0) as the electrophoresis buffer. The products were trans-

ferred to a nitrocellulose membrane, Hybond^{TM} ECL^{TM}, and the membrane was blocked using 5% non-fat dried milk. The streptavidin-peroxidase (1 mg/ml) was diluted 1,000 fold in PBST buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.3% Tween 20, pH 7.4), and incubated with the membrane for 2 h at room temperature. ECL chemi-luminescence was detected on an X-ray film using luminol as a substrate.

2.6. Incorporation of 5-bromo-2′-deoxyuridine (BrdU) into DNA in $CspA_{St}$-overproducing Cells

E. coli cells harboring pEAD4, pAED-cspA$_{St}$, or pFEAT30, were grown until the optical density at 600 nm (OD_{600}) reached ≈0.2, and then IPTG was added to a final concentration of 0.1 mM. Cultures were continued for 1 h to allow production of $CspA_{St}$, and 10 μM of BrdU, a deoxynucleoside analogue, was added. Cultures were progressed further for 4 h at 37°C, and cells were harvested by centrifugation and genomic DNA was purified according to the method of Ausubel *et al.* (1987). Incorporation of BrdU into genomic DNA was monitored using Cellular DNA fragmentation ELISA kit (Roche Applied Science), according to the manufacture's instruction (11585045001a.pdf) with slight modifications. Briefly, various amounts of genomic DNA were denatured using 0.25 N NaOH, and spotted on a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk in PBST buffer for 2 h. BrdU was detected using monoclonal mouse anti-BrdU antibody conjugated with peroxidase, and tetramethyl benzymidine was used as a substrate for color development.

3. RESULTS

3.1. Sequence Homology Between Bacterial Csps

To determine the molecular function of CspA from *Streptomyces* sp. AA8321 ($CspA_{St}$), we compared its amino acid sequence with known Csp sequences from various bacteria (Fig. 1). Csps are reported in more than 50 bacterial species and are highly conserved (Graumann and Marahiel, 1998). Highest degrees of homology were found with other *Streptomyces* Csp proteins; in particular, it shares 95% sequence identity with a Csp from *S. avermitilis* MA-4680. RNA-binding motif RNP1 of $CspA_{St}$ completely matches the consensus sequence

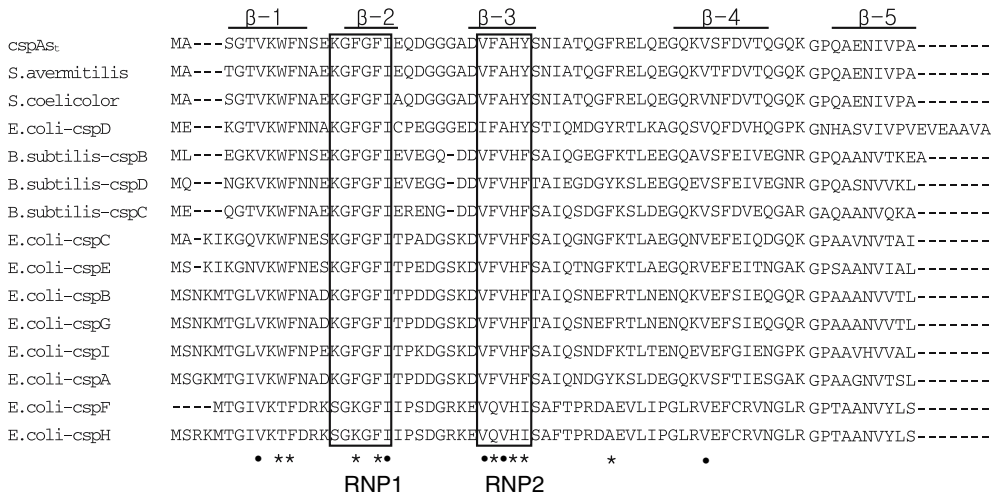


Fig. 1. Sequence alignment of $CspA_{St}$ protein with other representative bacterial Csp proteins. The Csp sequences were obtained from GenBank database and aligned with the default settings of CLUSTAL W (Thompson *et al.*, 1994). The Csp amino acid sequences analyzed in this study (accession number in parenthesis) were *S. avermitilis* (BAC72488), *S. coelicolor* A3(2) (CAB76995), *E. coli* cspD (ACC73967), *B. subtilis* cspB (NP_388791), *B. subtilis* cspC (NP_388393), *B. subtilis* cspD (NP_390076), *E. coli* cspA (ACC76580), *E. coli* cspB (BAA36131), *E. coli* cspC (NP_41637), *E. coli* cspE (ACC73724), *E. coli* cspF (BAA36130), *E. coli* cspG (NP_415510), *E. coli* cspH (NP_415509), and *E. coli* cspI (ACC74625). Gaps indicated by hyphens (-) were introduced to improve alignment, and 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 a hydrophobic core are indicated with solid circles (•) on the lowest line (Newkirk *et al.*, 1994). The five β-strands (β-1–β-5) are shown on the top line.

(Lys-Gly-Phe-Gly-Phe-Ile). The second RNA-binding motif RNP2 in $CspA_{St}$ is modified from the consensus sequence (Val-Phe-Val-His-Phe): with Val → Ala and Phe → Tyr substitutions at the central and terminal positions, respectively. The substitutions maintain the hydrophobicity and aromaticity characteristics of the original RNP2 motif.

Interestingly, the five hydrophobic residues (Val6, Ile18, Val27, Val29, and Val48; dots in Fig. 1) implicated in forming a hydrophobic core in Csps and the eight aromatic residues (Trp8, Phe9, Phe15, Phe17, Phe28, His30, Phe31, and Tyr39; asterisks in Fig. 1) that are critical in nucleic acid-binding by Csps (Newkirk *et al.*, 1994) are conserved for $CspA_{St}$. Therefore, our sequence analysis suggests that $CspA_{St}$ may contain a typical β-barrel cold-shock domain-fold and have single-stranded nucleic acid-binding activity, like other known Csps.

Since the functions of *E. coli* CspA and *E. coli* CspD are known, their sequences were compared to $CspA_{St}$ sequence. Sequence comparison reveals overall sequence homology, with 50 and 55% identity and 61 and 62% similarity to *E. coli* CspA and CspD, respectively. Thus, the sequence of $CspA_{St}$ is not sufficiently closer to one of them to allow specific functional assignment.

3.2. Overexpression of $CspA_{St}$ Prevents Colony Formation in *E. coli*

To study the role of $CspA_{St}$, the *cspA* gene from *Streptomyces* sp. AA8321 was cloned into the plasmid pAED4, which allows inducible expression in *E. coli* BL21(DE3) pLysS cells under control the of *lpp-lac* promoter. Overexpression of $CspA_{St}$ in *E. coli* was induced by addition of IPTG to mid-log-phase liquid cultures of cells carrying pAED-cspA$_{St}$. A small protein was abundantly expressed within 3 h of IPTG addition in cells harboring pAED-cspA$_{St}$, but not in cells harboring pAED4 (Fig. 2a).

When cell growth was assessed by optical density measurements of the culture at 600 nm, the cell mass continued to increase in $CspA_{St}$-overexpressing cells although at the lower levels than that of control cells carrying the pAED4 (Fig. 2b, top). The result showed that both transcription and translation continued to occur at moderate rates in $CspA_{St}$-expressing cells, since protein production, as reflected by optical density, continued to increase.

Surprisingly, the viability of $CspA_{St}$-overexpressing cells, as assessed by colony-forming ability, was less than 1% of that of pAED4 control cells (Fig. 2b, bottom). The result was visually confirmed on a solid medium (Fig. 2c). Whereas all the *E. coli* strains tested formed comparable numbers of colonies on a LB plate in the absence of IPTG, $CspA_{St}$-overexpressing cells formed far fewer colonies on a plate containing 0.1 mM IPTG.

To rule out the possibility that growth inhibition was a non-specific effect of heterologous protein overexpression, we compared these results to those from the same *E. coli* strain overexpressing human α_1-antitrypsin. Growth (Fig. 2b, top) and the colony-forming ability (Fig. 2b, bottom; Fig. 2c) of α_1-antitrypsin-overproducing cells were comparable to that of the pAED4 control cells.

When cell morphology was observed under a microscope, *E. coli* cells carrying the pAED4 vector were short and rod-shaped, whereas $CspA_{St}$-producing cells were elongated without a noticeable increase in cell width (Fig. 2d). The cellular morphology of α_1-antitrypsin-overproducing cells was indistinguishable from that of pAED4-carrying cells (Fig. 2d). When $CspA_{St}$ was expressed at 10°C or 42°C, *E. coli* cells continued to exhibit elongated morphology, even though at slightly lower levels than at 37°C. However, the expression levels of $CspA_{St}$ were also lower at these temperatures (data not shown). These phenotypes of $CspA_{St}$-overexpressing cells, impaired colony formation and elongated cell morphology, have a precedent: *E. coli* CspD-overexpressing cells have been reported previously to show similar phenotypes (Yamanaka *et al.*, 2001).

3.3. Purified $CspA_{St}$ Forms a Homodimer in Solution

After high-speed centrifugation of cellular extracts from $CspA_{St}$-overexpressing cells, most, if not all, of $CspA_{St}$ protein was found in the supernatant fraction. $CspA_{St}$ protein was purified by anion-exchange column chromatography and size-fractionation as described in Materials and methods. A single protein band with an apparent molecular mass of 7 kDa was observed on SDS-PAGE (Fig. 3a), as visualized by staining with Coomassie Brilliant Blue. The molecular mass determination of the purified protein using a Shimadzu AXIMA-LNR MALDI-TOF mass spectroscope and *N*-terminal amino acid sequencing using Milligen 6600 (Applied Biosystems) confirmed that the purified protein is indeed $CspA_{St}$. The purified protein exhibited a circular dichroism spectrum

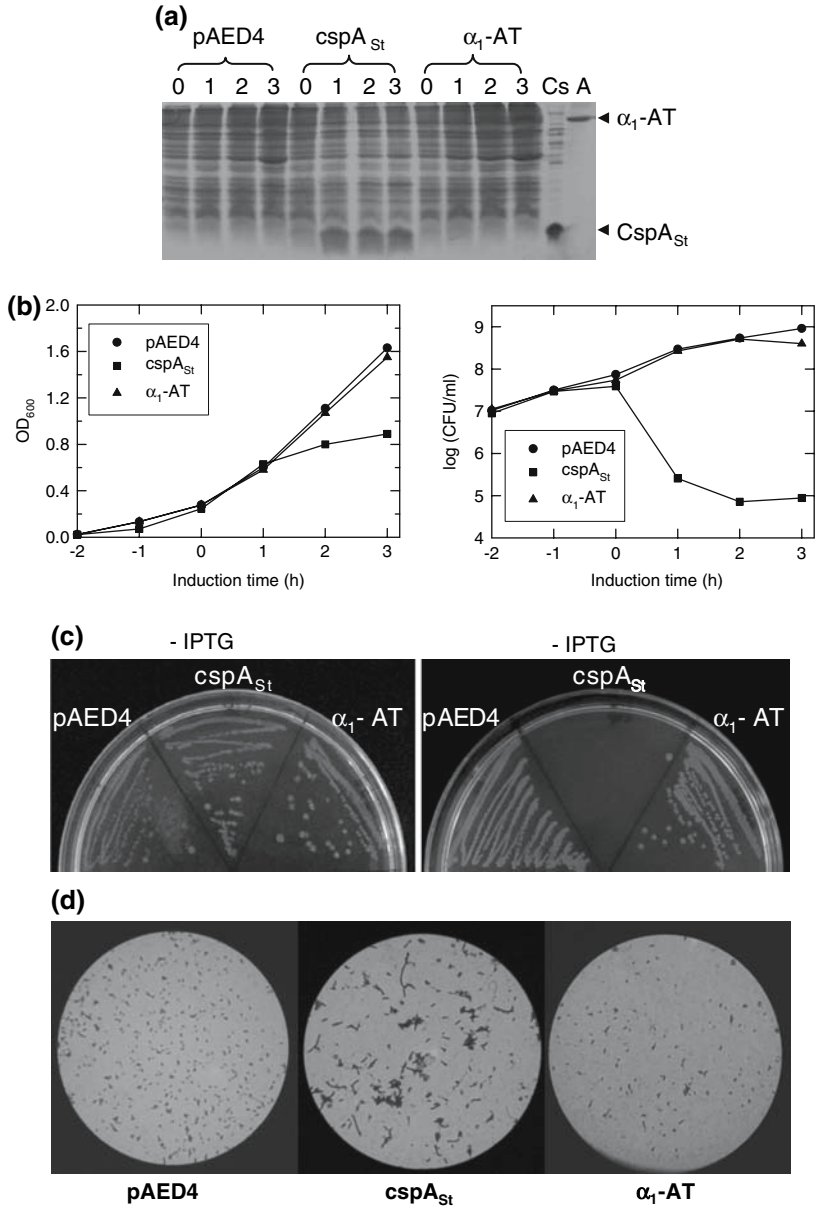


Fig. 2. Effects of $CspA_{St}$-overexpression on colony formation and cellular morphology. (a) SDS-polyacrylamide gel electrophoresis showing the overexpression of $CspA_{St}$. *E. coli* BL21(DE3) pLysS cells carrying pAED-cspA$_{St}$ were incubated at 37°C until OD_{600} reaches ≈0.2, and $CspA_{St}$ overexpression was induced by addition of 0.1 mM IPTG. One ml cultures were taken at each time points after IPTG addition, and expression pattern was analyzed on 20% SDS-polyacrylamide gel electrophoresis. As a control, *E. coli* BL21(DE3) pLysS cells carrying pAED4 (vector) or pFEAT30 (for expression of recombinant α_1-antitrypsin; α_1-AT) were sampled using the same method. Lanes: 0, 1, 2, 3, h after IPTG addition; Cs, partially purified $CspA_{St}$; A, purified α_1-AT. (b) Effects of $CspA_{St}$-overexpression on cell growth (left graph) and cell viability (right graph). OD_{600} and the colony forming units (CFU) of the cultures were measured every hour. (c) Effects of $CspA_{St}$-overexpression on colony formation. Overnight cultures of *E. coli* cells were streaked on a solid medium without added IPTG (left) or with 0.1 mM IPTG (right), and incubated at 37°C for 16 h. (d) Effects of $CspA_{St}$-overexpression on cellular morphology. Cells were fixed at 3 h after addition of IPTG, stained with safranin, and observed at 1,000× magnifications.

typical of β-strand-dominated proteins, as previously reported for other Csp proteins (data not shown). Conservation of the five hydrophobic residues implicated in formation of a hydrophobic core in Csps also suggests that $CspA_{St}$ adopts a cold-shock domain-fold structure.

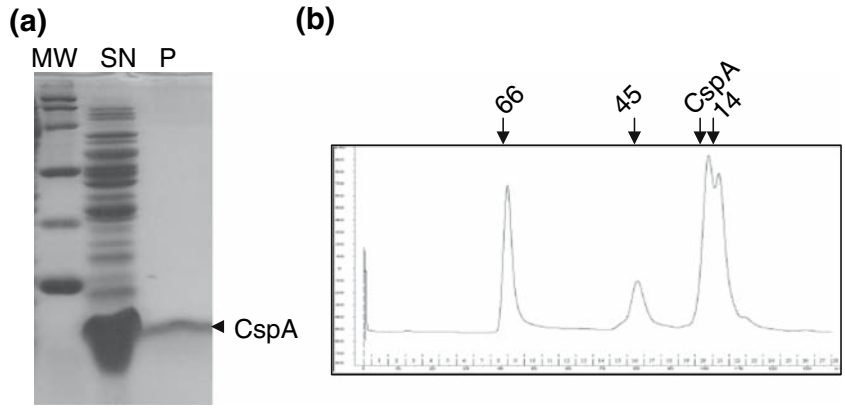


Fig. 3. Characterization of the purified $CspA_{St}$. (a) Purification of $CspA_{St}$ is followed by 20% SDS-polyacrylamide gel electrophoresis. $CspA_{St}$ protein in cell lysate was purified by an anion-exchange column chromatography and a size-fractionation. MW, molecular mass standard (Gibco BRL, Gaitherburg, USA; from the top, 200 kDa, 97.4 kDa, 68 kDa, 43 kDa, 29 kDa, and 18.4 kDa); SN, supernatant fraction of cell lysate; P, purified $CspA_{St}$ protein. (b) Gel filtration of $CspA_{St}$ protein on a Superdex 75 column. $CspA_{St}$ protein (1 mg/ml) was applied onto a FPLC Superdex 75 column pre-equilibrated with 20 mM Tris–HCl buffer, pH 8.0. The flow rate was 1 ml/min, and protein-elution was monitored by UV absorbance at 214 nm. Molecular size standards were bovine serum albumin (66 kDa), recombinant human α_1-antitrypsin (44 kDa), and lysozyme (14 kDa) in the same buffer.

Gel filtration of $CspA_{St}$ on a Superdex-75 column resulted in an elution profile consistent with that of a 15-kDa protein (Fig. 3b), indicating that $CspA_{St}$ exists exclusively as a homodimer under native conditions. Although the best-studied Csp, *E. coli* CspA, is found as a monomer in a solution, CspD of *E. coli* (Yamanaka *et al.*, 2001) and CspB of *B. subtilis* (Schindelin *et al.*, 1993) are reported to have a homodimeric conformation. In the crystal structure of *B. subtilis* CspB, antiparallel β4-β4 intermolecular interactions were observed (Schindelin *et al.*, 1993). Notably, the dimer-forming Csps ($CspA_{St}$, *E. coli* CspD, and *B. subtilis* CspB) are three amino acids shorter at their *N*-termini than the monomeric *E. coli* CspA. It has been suggested that the extended *N*-terminus of the latter may interact intramolecularly with the β4-strand, preventing the intermolecular association necessary for dimerization (Newkirk *et al.*, 1994).

3.4. $CspA_{St}$ Binds ssDNA but not Double-stranded DNA (dsDNA)

Since the RNP-1 and RNP-2 sequence motifs are conserved in $CspA_{St}$, we examined the DNA-binding activity of the protein. In gel-shift assays with heat-denatured ssDNA as a probe, addition of purified $CspA_{St}$ retarded migration of the probe band (Fig. 4, left). Increases in $CspA_{St}$ concentration in the binding reaction resulted in increased retardation of the probe in a concentration-dependent manner (Fig. 4, left). $CspA_{St}$ also exhibited binding affinity for small ssDNA, as shown by a gel

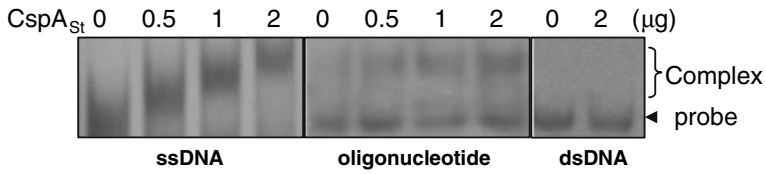


Fig. 4. DNA binding activity of $CspA_{St}$. (left) Fifty ng of ssDNA was incubated with various amounts of $CspA_{St}$, as indicated on the top of the figure, on ice for 20 min, and the reaction products were analyzed on 8% polyacrylamide gels. (center) Various amounts of $CspA_{St}$ were incubated with 100 picomol of 5′-botynylated primers of sequence 5′-TCGAGTGCGGCCGCAAGCTT-3′ on ice for 20 min, and the reaction products were analyzed on 12% polyacrylamide gel. (right) Fifty ng of double-stranded DNA was incubated with 2 μg of $CspA_{St}$ on ice for 20 min, and the reaction products were analyzed on 8% polyacrylamide gels.

shift with a 20-nucleotide probe (Fig. 4, middle). In contrast, dsDNA did not gel-shift upon addition of $CspA_{St}$ (Fig. 4, right). These results indicate that $CspA_{St}$ binds to ssDNA but not to dsDNA.

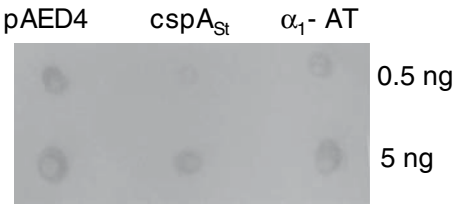
3.5. Incorporation of BrdU into DNA is Inhibited by Overexpression of $CspA_{St}$

Since the ability of $CspA_{St}$ to bind ssDNA and the homologous characteristics to *E. coli* CspD suggested a role for $CspA_{St}$ in DNA replication, we monitored incorporation of BrdU, a deoxynucleoside analogue, into genomic DNA of $CspA_{St}$-overexpressing cells. When 0.5 ng of genomic DNA was used, incorporation of BrdU was negligible in $CspA_{St}$-producing cells, whereas control cells carrying the vector only or overexpressing α_1-antitrypsin rapidly incorporated the nucleoside (Fig. 5). When ten times as much genomic DNA was used, BrdU incorporation into $CspA_{St}$-overproducing cells was barely detectable. Densitometric scanning results demonstrate that DNA synthesis in the $CspA_{St}$-overproducing cells was about 4% that of control cells. This result supports that DNA replication is severely impaired in $CspA_{St}$-overproducing cells.

4. DISCUSSION

In this study, we overproduced and characterized $CspA_{St}$ of the polar bacterium, *Streptomyces* sp. AA8321. Overproduction of $CspA_{St}$ inhibited host-cell colony formation by more than 99%, as compared to control cells carrying the pAED4 vector alone (Fig. 2). Furthermore, the ability of purified $CspA_{St}$ to bind ssDNA and the inability of CspA-overexpressing cells to incorporate BrdU into DNA strongly suggest that $CspA_{St}$ affects DNA replication. Even though we could not rule out some indirect effects of overproduction of heterologous proteins on DNA replication, lack of similar effects in α_1-antitrypsin overproducing cells suggests that it is less likely. To our best knowledge, this is the first report elucidating the function of Csp from a polar bacterium, and the second case of Csps functioning as a DNA replication inhibitor: purified *E. coli* CspD has been reported to block DNA synthesis in replication system *in vitro* (Yamanaka *et al.*, 2001). $CspA_{St}$ and *E. coli* CspD are also similar in that each forms a homodimer under physiological conditions and they each bind to ssDNA in a concentration-dependent manner. These characteristics are in contrast to those of *E. coli* CspA, which functions as an RNA chaperone, exists as a monomer, and binds ssDNA cooperatively. Overproduction of *E. coli* CspD also inhibits colony formation, and induces an elongated morphology. Interestingly, although $CspA_{St}$ exhibits overall sequence homology to both CspA and CspD of *E. coli* (61.4 and 62.2% similarity, respectively), its RNP-1 and RNP-2 domain sequences are more similar to those of *E. coli* CspD than to those of *E. coli* CspA. The result also favors a role for $CspA_{St}$ as a DNA replication inhibitor, although mutational analysis is needed to elucidate the contribution of RNP sequences on DNA replication.

Because *Streptomyces* sp. AA8321 is an Antarctic bacterium, it is well adapted to cold stress. Although the contribution of other factors in cold tolerance, such as fatty acid desaturases, protein chaperones, or increased osmomolarity, needs to be evaluated in this bacterium, this study suggest a role for $CspA_{St}$ in halting DNA replication until the cell adjusts itself upon sudden temperature drops. In our $CspA_{St}$-overproduction experiments, the amounts of $CspA_{St}$ protein declines at 5 h after induction, and the cell viability resumes simultaneously (data not shown). Since *Streptomyces* sp. AA8321 has three additional *csp* genes, other Csps may contribute to cold tolerance in this bacterium as well.



	pAED4	cspA St	α1-AT
R.A.	1.00	0.04	0.78

Fig. 5. Impaired incorporation of BrdU into DNA in $CspA_{St}$-overexpressing cells. Various amounts of genomic DNA from host cells were spotted on a nitrocellulose membrane, and incorporated BrdU was detected using a monoclonal anti-BrdU antibody conjugated to peroxidase, as described in Materials and methods. Densitometric scanning results are presented as relative amounts (R.A.).

ACKNOWLEDGMENTS

This work was supported from grant number R01-2006-000-11154-0 from the Basic Research

Program of the Korea Science & Engineering Foundation, Grant PM05090 from the Korea Ministry of Maritime Affairs and Fisheries, and grant number FPR05B2-211 of 21C Frontier Functional Proteomics Program from the Korea Ministry of Science and Technology.

REFERENCES

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) Current Protocols in Molecular Biology. New York: Greene Publishing Associates and Wiley-Interscience.

Av-Gay, Y., Aharonowitz, Y., and Cohen, G. (1992). *Nucleic Acids Res*. **20:** 5478.

Bentley, S. D., Chater, K. F., Cerdeno-Tarraga, A. M., Challis, G. L., Thomson, N. R., James, K. D., Harris, D. E., Quail, M. A., Kieser, H., Harper, D., Bateman, A., Brown, S., Chandra, G., Chen, C. W., Collins, M., Cronin, A., Fraser, A., Goble, A., Hidalgo, J., Hornsby, T., Howarth, S., Huang, C. H., Kieser, T., Larke, L., Murphy, L., Oliver, K., O'Neil, S., Rabbinowitsch, E., Rajandream, M. A., Rutherford, K., Rutter, S., Seeger, K., Saunders, D., Sharp, S., Squares, R., Squares, S., Taylor, K., Warren, T., Wietzorrek, A., Woodward, J., Barrell, B. G., Parkhill, J., and Hopwood, D. A. (2002). *Nature* **417:** 141–147.

Doering, D. (1992). Functional and Structural Studies of a Small F-actin Binding Domain, Ph.D. thesis, Massachusetts Institute of Technology, MA.

Ermolenko, D. N., and Makhatadze, G. I. (2002). *Cell. Mol. Life Sci*. **59:** 1902–1913.

Graumann, P., Schroder, K., Schmid, R., and Marahiel, M. A. (1996). *J. Bacteriol*. **178:** 4611–4619.

Graumann, P. L., and Marahiel, M. A. (1998). *Trends Biochem. Sci*. **23:** 286–290.

Ikeda, H., Ishikawa, J., Hanamoto, A., Shinose, M., Kikuchi, H., Shiba, T., Sakaki, Y., Hattori, M., and Omura, S. (2003). *Nat. Biotechnol*. **21:** 526–531.

Im, H., Woo, M.-S., Hwang, K. Y., and Yu, M.-H. (2002). *J. Biol. Chem*. **277:** 46347–46354.

Jiang, W., Hou, Y., and Inouye, M. (1997). *J. Biol. Chem*. **272:** 196–202.

Jones, P. G., VanBogelen, R. A., and Neidhardt, F. C. (1987). *J. Bacteriol*. **169:** 2092–2095.

Kormanec, J., and Ševčíková, B. (2000). *Mol. Gen. Genet*. **264:** 251–256.

Kremer, W., Schuler, B., Harrieder, S., Geyer, M., Gronwald, W., Welker, C., Jaenicke, R., and Kalbitzer, H. R. (2001). *Eur. J. Biochem*. **268:** 2527–2539.

Landsman, D. (1992) *Nucleic Acids Res*. **20:** 2861–2864.

Lopez, M. M., and Makhatadze, G. I. (2000). *Biochem. Biophys. Acta* **1479:** 196–202.

Martínez-Costa, O.-H., Zalacaín, M., Holmes, D. J., and Malpartida, F. (2003). *FEMS Microbiol. Lett*. **220:** 215–221.

Mikulík, K., Khanh-Hoang, Q., Halada, P., Bezouskova, S., Benada, O., and Behal, V. (1999). *Biochem. Biolphys. Res. Commun*. **265:** 305–310.

Nakashima, K., Kanamaru, K., Mizuno, T., and Horikoshi, K. (1996). *J. Bacteriol*. **178:** 2994–2997.

Newkirk, K., Feng, W., Jiang, W., Tejiro, R., Emerson, S. D., Inouye, M., and Montelione, G. T. (1994). *Proc. Natl. Acad. Sci. USA* **91:** 5114–5118.

Omura, S., Ikeda, H., Ishikawa, J., Hanamoto, A., Takahashi, C., Shinose, M., Takahashi, Y., Horikawa, H., Nakazawa, H., Osonoe, T., Kikuchi, H., Shiba, T., Sakaki, Y., and Hattori, M. (2001). *Proc. Natl. Acad. Sci. USA* **98:** 12215–12220.

Phadtare, S., Hwang, J., Severinov, K., and Inouye, M. (2003). *Genes Cells* **8:** 801–810.

Phadtare, S. (2004) *Curr. Issues Mol. Biol*. **6:** 125–136.

Schindelin, H., Marahiel, M. A., and Heinemann, U. (1993). *Nature* **364:** 164–168.

Schindelin, H., Jiang, W., Inouye, M., and Heinemann, U. (1994). *Proc. Natl. Acad. Sci. USA* **91:** 5119–5123.

Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). *Nucleic Acids Res*. **22:** 4673–4680.

Wang, N., Yamanaka, K., and Inouye, M. (1999). *J. Bacteriol*. **181:** 1603–1609.

Xia, B., Ke, H., and Inouye, M. (2001). *Mol. Microbiol*. **40:** 179–188.

Yamanaka, K., and Inouye, M. (1997). *J. Bacteriol*. **179:** 5126–5130.

Yamanaka, K., Fang, L., and Inouye, M. (1998). *Mol. Microbiol*. **27:** 247–255.

Yamanaka, K., Zheng, W., Crooke, E., Wang, Y. H., and Inouye, M. (2001). *Mol. Microbiol*. **39:** 1572–1584.