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Structure and Function of Cold Shock Proteins in Archaea▽

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Archaea are abundant and drive critical microbial processes in the Earth's cold biosphere. Despite this, not enough is known about the molecular mechanisms of cold adaptation and no biochemical studies have been performed on stenopsychrophilic archaea (e.g., *Methanogenium frigidum*). This study examined the structural and functional properties of cold shock proteins (Csps) from archaea, including biochemical analysis of the Csp from *M. frigidum*. *csp* genes are present in most bacteria and some eucarya but absent from most archaeal genome sequences, most notably, those of all archaeal thermophiles and hyperthermophiles. In bacteria, Csps are small, nucleic acid binding proteins involved in a variety of cellular processes, such as transcription. In this study, archaeal Csp function was assessed by examining the ability of *csp* genes from psychrophilic and mesophilic *Euryarchaeota* and *Crenarchaeota* to complement a cold-sensitive growth defect in *Escherichia coli*. In addition, an archaeal gene with a cold shock domain (CSD) fold but little sequence identity to Csps was also examined. Genes encoding Csps or a CSD structural analog from three psychrophilic archaea rescued the *E. coli* growth defect. The three proteins were predicted to have a higher content of solvent-exposed basic residues than the noncomplementing proteins, and the basic residues were located on the nucleic acid binding surface, similar to their arrangement in *E. coli* CspA. The *M. frigidum* Csp was purified and found to be a single-domain protein that folds by a reversible two-state mechanism and to exhibit a low conformational stability typical of cold-adapted proteins. Moreover, *M. frigidum* Csp was characterized as binding *E. coli* single-stranded RNA, consistent with its ability to complement function in *E. coli*. The studies show that some Csp and CSD fold proteins have retained sufficient similarity throughout evolution in the *Archaea* to be able to function effectively in the *Bacteria* and that the function of the archaeal proteins relates to cold adaptation. The initial biochemical analysis of *M. frigidum* Csp has developed a platform for further characterization and demonstrates the potential for expanding molecular studies of proteins from this important archaeal stenopsychrophile.

Studies investigating the ecology and biology of cold-adapted archaea have flourished in the last 10 years, particularly in response to the developing understanding of the critical roles archaea play in the cold biosphere (8). The psychrophilic archaeon with the lowest known upper growth temperature limit (18°C) is *Methanogenium frigidum*, an H_2-CO_2-utilizing methanogen isolated from methane-saturated waters in Ace Lake, Antarctica (17). Because of its restricted low-temperature growth range (it is a stenopsychrophile) in comparison to other methanogens, which are capable of growth at temperatures of up to 110°C, *M. frigidum* has served as a critical resource for comparative genomic studies investigating the basis of thermal adaptation in archaea (45). Despite its unique stenopsychrophilic properties, biochemical studies of proteins from *M. frigidum* have not been reported.

One of the few genes from the draft genome of *M. frigidum* that were noted for their possible roles in cold adaptation is the *csp* gene, which is predicted to encode a small (7.8-kDa) acidic nucleic acid binding protein (45). Csps are a hallmark of the *Bacteria* and are synthesized under a range of growth conditions, most notably during cold growth or following cold shock, where they may represent up to 10^6 molecules per cell (54). Genes with a high degree of similarity to the *Escherichia coli* gene *cspA* are present in the genomes of many organisms, including psychrophilic, mesophilic, and thermophilic bacteria (11, 32, 60, 62), yeasts, slime molds, plants, and animals (27, 34, 44, 56).

In archaea, *csp* homologs have been identified in DNA sequences from only a small number of psychrophiles and mesophiles and do not appear to be present in thermophiles or hyperthermophiles (8). In addition to *M. frigidum csp*, homologs have been identified in the genome sequences of haloarchaea including the Antarctic haloarchaeon *Halorubrum lacusprofundi* (described in this study) (42) and the mesophiles *Haloferax volcanii*, *Halobacterium* sp. strain NRC-1 (8, 17, 45), and *Haloarcula marismortui* (described in this study). In addition to these members of the *Euryarchaeota*, *csp* genes have been reported in the marine *Crenarchaeota* member "*Cenarchaeum symbiosum*" (9) and uncultured deep sea planktonic *Crenarchaeota* (3).

Csps share high sequence similarity with the nucleic acid binding domains of eucaryal Y-box proteins (22, 61). These

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domains contain a protein fold described as the cold shock domain (CSD) fold, which consists of a five-stranded antiparallel β-barrel capped by a long, flexible loop (29). The CSD contains two RNA binding motifs, RNP1 and RNP2, that are involved in binding to RNA and DNA. CSDs have also been identified in eucaryal proteins that do not belong to the Y-box family (24, 26). Moreover, the minor allergen protein Cla h 8, from the ascomycete fungus *Cladosporium herbarum*, consists solely of a CSD, similar to bacterial Csps (14). Cla h 8 shows 76% amino acid identity with *E. coli* CspA, although it displays nucleic acid binding properties more similar to those of the eucaryal CSD, and Cla h 8 has been suggested to represent an evolutionary link between bacterial Csps and eucaryal CSDs (14).

E. coli encodes nine *csp* genes (*cspA* to *cspI*), of which *cspA*, *cspB*, *cspG*, and *cspI* are induced by cold shock (13, 19, 31, 37, 58). In contrast, the *cspC* and *cspE* genes are constitutively expressed, *cspD* is induced by nutritional deprivation, and the expression of *cspF* and *cspH* has not been associated with any particular growth condition or phenotype (2, 64, 65). In a strain with three of the cold-induced genes (*cspA*, *cspB*, and *cspG*) deleted, the CspE protein was found to be overproduced when cells were grown at low temperature (2). By deleting *cspE*, in addition to *cspA*, *cspB*, and *cspG*, a cold-sensitive strain, BX04, was generated that was unable to form colonies at 15°C (63). The cold sensitivity of BX04 can be suppressed by overexpressing any of the *E. coli csp* genes, except *cspD*.

The growth defect of BX04 was also complemented by the S1 domain of polynucleotide phosphorylase (PNPase) (7). The S1 domain is a CSD fold domain found in ribosomal protein S1 and in a large number of other RNA-associated proteins (6, 35). Unlike *M. frigidum*, the Antarctic methanogen *Methanococcoides burtonii* does not encode a *csp* homolog. However, two hypothetical proteins predicted to have CSD folds were identified in the draft genome sequence, and the best threading template for the two hypothetical proteins is the S1 domain of PNPase (45).

The presence of bacterial Csp homologs in *Euryarchaeota* (methanogens and haloarchaea) and *Crenarchaeota* (uncultured planktonic and symbiotic marine archaea) from cold environments, and in mesophilic *Euryarchaeota* (haloarchaea) raises questions about their role in low-temperature adaptation in the *Archaea*. To begin to determine the role of archaeal Csps and structural analogs with CSD folds, we designed a series of in vivo complementation studies of *E. coli* with representative genes from methanogens, halophiles, and *Crenarchaeota* and initiated biochemical studies on *M. frigidum* Csp to probe its structure and function. The studies have achieved a range of new insights into the roles of a new class of archaeal genes and have developed a platform for heterologous biochemical studies of *M. frigidum* proteins.

MATERIALS AND METHODS

PCR amplification, cloning, and sequencing. *M. frigidum csp* was amplified from an *M. frigidum* genomic library (45). *csp* genes were identified from available genome sequence data on *H. marismortui* (4), *H. lacusprofundi* (20), and *Halobacterium* sp. strain NRC-1 (12) and amplified from genomic DNA. "*C. symbiosum*" *csp* and uncultured *Crenarchaeota csp* genes were amplified from fosmids C7E8 and 4B7 (3). The *M. burtonii* CSD gene was amplified from *M. burtonii* genomic DNA. PCRs were carried out in a total volume of 50 μl containing 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate, 2.5 U *Taq* DNA polymerase (Sigma), 1× *Taq* DNA polymerase buffer, 20 pmol of forward and reverse primers (sequences are available upon request), and approximately 50 ng of DNA templates. Forward primers were designed to introduce an NdeI restriction site overlapping the ATG initiation codon. Reverse primers were designed to generate either a BamHI restriction site for cloning into green fluorescent protein (57) and pET-15b (Novagen) vectors or a SapI restriction site for cloning *M. frigidum csp* into pTYB1 (New England BioLabs). PCRs were carried out in a Hybaid Thermal Cycle PCR machine for 30 cycles (94°C for 30 s, 55°C for 60 s, and 72°C for 90 s) after initial denaturation at 96°C for 60 s. The amplified products were gel purified with a Prep-A-gene kit (Bio-Rad) and ligated into a pGEM-T cloning vector (Promega) according to the manufacturer's instructions. Recombinant plasmids harboring the *csp* genes and *M. burtonii* CSD were double digested with NdeI and BamHI and cloned between the same two sites of a green fluorescent protein vector. The new constructs were digested with XbaI and BamHI, and *csp* genes were subcloned into a modified pIN-III expression vector, pIN-III-$lpp^{P\text{-}5}$. *M. frigidum csp* was also cloned into pTYB1 and pET-15b expression vectors between the NdeI/SapI and NdeI/BamHI restriction sites, respectively. In the former, *M. frigidum csp* was fused to the N terminus of an intein-chitin binding domain tag, whereas in the latter *M. frigidum csp* was fused to the C terminus of a His tag. All recombinant plasmids were transformed into chemically competent *E. coli* strain DH5α cells. Constructs were verified by DNA sequencing (ABI 377 DNA sequencer, Automated DNA Analysis Facility, The University of New South Wales).

Complementation testing and expression analysis of archaeal genes in *E. coli* BX04. pIN-III-$lpp^{P\text{-}5}$ plasmids harboring the archaeal *csp* genes and *M. burtonii* CSD were transformed into *E. coli* strain BX04 (63). Cells were grown in LB medium supplemented with ampicillin (100 μg ml^{-1}) at 37°C to an optical density at 600 nm of 0.6. Gene expression was induced by addition of 1, 2, 10, or 70 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and cultures were incubated at 15°C for 16 h. Cells were harvested at 4°C and resuspended in 20 mM Tris HCl (pH 7.5)–200 mM NaCl–1 mM EDTA–0.5 to 1 mg of lysozyme. Cells were lysed on ice by sonication with a Branson Sonifier for four to six cycles of 30 s on a 35% duty cycle at a power setting of 3. Soluble and insoluble fractions were separated by centrifugation at 12,000 rpm at 4°C and analyzed by 16% (wt/vol) sodium dodecyl sulfate (SDS)-gel electrophoresis. To assess complementation ability, single colonies of recombinant BX04 cells were inoculated into LB medium and grown at 37°C for 4 to 5 h. Cultures (2 μl) were streaked onto LB agar plates supplemented with ampicillin (100 μg ml^{-1}) or with ampicillin plus IPTG (1, 2, 10, or 70 mM), and plates were incubated at 4, 10, 15, 23, and 30°C. Plates were incubated for various lengths of time (depending on the temperature), and growth of BX04 with a plasmid backbone only (control) was compared with that of strain BX04 harboring the archaeal *csp* genes and *M. burtonii* CSD.

Overexpression and purification of *M. frigidum* Csp. *M. frigidum csp* cloned into pTYB1 was transformed into *E. coli* strain BL21(DE3) [F^- *ompT* $hsdS_B$ (r_B^- m_B^-) *gal dcm*(DE3)]. Cells were grown at 37°C in LB medium supplemented with ampicillin (100 μg ml^{-1}) to an optical density at 600 nm of 0.5 to 0.6. Gene expression was induced by the addition of 1 mM IPTG, and the culture was incubated at 15°C for approximately 16 h. Cells were harvested by centrifugation at 4°C at 5,000 × *g* for 30 min and resuspended in 1/15 culture volume of lysis buffer (20 mM Tris HCl [pH 7.5], 1 mM EDTA). Cells were lysed by sonication as described above. Soluble and insoluble fractions were separated by centrifugation at 12,000 × *g* at 4°C and analyzed by SDS-gel electrophoresis. *M. frigidum* Csp was purified on a chitin column by a procedure that was described previously (55), with minor modifications. The supernatant was loaded onto a chitin bead column (1/50 culture volume) preequilibrated with 10 bed volumes of column buffer (20 mM Tris HCl, 1 mM EDTA, 200 mM NaCl). The column was washed with 20 bed volumes of column buffer with a linear gradient of NaCl (200 mM → 950 mM → 200 mM). The cleavage of the fusion protein was induced by flushing the column with 3 bed volumes of column buffer containing 70 mM dithiothreitol (DTT). The reaction mixture was incubated at 22°C for 64 h, and the protein was eluted in column buffer. *M. frigidum* Csp concentration was determined by Bradford assay (5), and amino acid analysis was performed at the Australian Proteome Analysis Facility, Macquarie University, Sydney, Australia. Protein purity was determined by visualization of protein bands on SDS-gels.

TUG-GE. Transverse urea gradient gel electrophoresis (TUG-GE) was performed as described previously (10, 53). Gels were prepared with a urea gradient of 0 to 7 M and an inverse acrylamide gradient of 15 to 11%. The bottom solution (35 ml) consisted of 0.375 M Tris-HCl (pH 8.8), 7 M urea, 11% acrylamide solution (40% acrylamide, 3.3% bisacrylamide), 0.026% (vol/vol) *N,N,N′,N′*-tetraethylenediamine (TEMED), and 0.015% (wt/vol) ammonium persulfate. Gradient solutions (57.5 ml each) consisted of 0.375 M Tris-HCl (pH 8.8), 7 M or 0 M urea (gradient solution with or without urea, respectively), 11% or 15%

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TABLE 1. Amino acid identities of Csp homologs from *H. marismortui* and *H. lacusprofundi*

Organism and gene	% Identity								
	380453	260063	260061	380451	390287	846	1693	1547	825
H. marismortui									
380453	100	95	88	89	84				
260063		100	89	88	83				
260061			100	89	78				
380451				100	84				
390287					100				
H. lacusprofundi									
846						100	89	81	83
1693							100	82	86
1547								100	81
825									100

acrylamide solution (gradient solution with or without urea, respectively), 0.026% (vol/vol) TEMED, and 0.013% (wt/vol) ammonium persulfate. The top solution (36 ml) consisted of 0.375 M Tris-HCl (pH 8.8), 0 M urea, 15% acrylamide solution, 0.026% (vol/vol) TEMED, and 0.013% (wt/vol) ammonium persulfate. *M. frigidum* Csp samples (total volume, 75 μl) contained 30 μg of purified protein, 50 mM Tris HCl (pH 8.8), 0.005% bromophenol blue, and either 10% glycerol (N⇆U transition) or 7 M urea (U⇆N transition). The latter sample was preincubated for 2 h at 7°C before commencing electrophoresis. Gels were assembled in a Hoefer SE-250 electrophoresis unit (Bio-Rad) connected to a temperature-controlled water bath (MultiTemp III; Pharmacia Biotech) set at 1°C and prerun at 10 mA for 30 min. The internal temperature was 7 to 10°C. Electrophoresis was performed in 0.3% (wt/vol) Tris–1.9% (wt/vol) glycine buffer (pH 8.3) at 10 mA for approximately 3 h. Gels were washed in Milli-Q water and stained with silver, and gel images were collected on a LAS3000 (Fujifilm, Melbourne, Australia) with ImageGauge v4.0 software. The conformational stability of *M. frigidum* Csp (ΔG [stability of the native conformation relative to that of the unfolded state] and $[\text{urea}]_{1/2}$ [urea concentration at $\Delta G = 0$]) was calculated as described previously (10, 18, 53).

Matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). All samples were purified with C_{18} ZipTips (Millipore) and mixed with a matrix solution prepared as follows. 3-Hydroxypicolinic acid (3-HPA) was dissolved in 50% CH_3CN (50 mg/ml) and mixed 9:1 with a solution of ammonium citrate (50 mg/ml in Milli-Q water). Mixtures were spotted onto a steel MALDI target and allowed to air dry. MALDI-TOF mass spectra were acquired in linear mode on a Voyager DE STR mass spectrometer (PE Biosystems) with manual acquisition control (200 laser shots/spectrum), a 25-kV accelerating voltage, a 94.2% grid voltage, and a 125-ns extraction delay time. By this approach, a mass error of about ±2 Da was observed, allowing easy discrimination of most of the ribonucleotide base residue masses, except cytidine and uridine, the residue masses of which are 305.18 Da and 306.17 Da, respectively.

Homology modeling. Models were generated with the protein homology modeling server SWISS-MODEL, and models were manipulated with the Swiss-Pdb Viewer.

Archaeal Csp models were predicted to form a β-barrel structure homologous to the bacterial Csp structures. Similar models for the *M. burtonii* CSD were generated previously (45).

RESULTS

Selection of archaeal Csp homologs. Five Csp sequences from *H. marismortui* with high identity (78 to 95%) to each other and four from *H. lacusprofundi* (81 to 89%) were identified (Table 1). The *H. marismortui* gene with the highest similarity to other *H. marismortui* genes (*H. marismortui* contig 380453) was chosen for complementation studies. The *H. lacusprofundi* genes with the greatest (*H. lacusprofundi* contig 846) and least (*H. lacusprofundi* contig 1547) similarity to other *H. lacusprofundi csp* genes were chosen for complementation. The two *csp* genes from *Halobacterium* sp. strain NRC-1 (genes 101 and 1836), one *csp* gene from uncultured *Crenarchaeota*, one gene from "*C. symbiosum*", and *M. frigidum csp* were chosen, providing a total of eight archaeal *csp* genes. Additionally, an *M. frigidum csp* construct with a His tag fused to the N terminus (*M. frigidum csp*-His) of the protein was included in the complementation assays. One of the two *M. burtonii* hypothetical proteins with a predicted CSD fold (*M. burtonii* CSD) was also chosen for complementation.

The similarity between the eight archaeal Csps and *E. coli* CspA varies between 36 and 59% amino acid identity (Table 2). *M. frigidum* Csp has higher similarity to *E. coli* CspA (59%) than to any of the other archaeal proteins (36 to 44%). The five haloarchaeal proteins are most similar to each other (68 to 91%), and the two Csps from *Crenarchaeota* are most similar to each other (73%). The *M. burtonii* CSD has very low sequence identity (5 to 7%) to any of the Csps.

Complementation of cold sensitivity in *E. coli* by archaeal genes. The archaeal genes were cloned into IPTG-inducible, high-level expression plasmid pIN-III-lpp^{P-5} in *E. coli* BX04, and growth was assessed on plates incubated at 30, 23, and 15°C. In the presence or absence of IPTG, BX04 pIN-III-lpp^{P-5} only formed colonies at 30°C (Fig. 1). In contrast, the strain harboring *M. frigidum csp* formed colonies at 23 and 15°C (Fig. 1). Similar patterns of complementation were achieved with IPTG concentrations of 1, 2, 10, and 70 mM (data not shown), and complementation only occurred in the presence of inducer (Fig. 1). When plates were incubated at 10 and 4°C, colonies did not form (data not shown). The fact that the *M. frigidum csp* complements the growth defect of the quadruple-deletion strain and suppresses its cold sensitivity at 15 and 23°C demonstrates that this protein from an archaeal psychrophile is biologically active and able to fulfill a critical functional role in a mesophilic bacterial host.

The ability of other archaeal genes to complement the cold sensitivity of BX04 was assessed at 15°C (data not shown). The uncultured *Crenarchaeota csp* complemented BX04 at 15°C in both the presence and the absence of IPTG. Similarly, when *M. frigidum csp*-His was cloned into a pIN vector and expressed in BX04, growth occurred in both the presence and the absence of IPTG. The ability to complement in the absence of inducer may reflect the ability of pIN vectors to allow basal expression of cloned genes even in the absence of inducer (63). It is not clear whether the different complementation patterns for un-

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induced BX04 with *M. frigidum csp*-His compared to *M. frigidum csp* relates to differential gene expression levels, stability of the proteins, or functionality mediated by the His tag. However, the fact that *M. frigidum csp*-His is functional in vivo encourages its use for future in vitro studies.

The *M. burtonii* CSD construct enabled colonies to form at 15°C only when plates did not contain IPTG (data not shown). This may indicate that the *M. burtonii* CSD functions effectively in *E. coli* when present at a low concentration in the cell, whereas at higher concentrations it prevents cell growth.

None of the haloarchaeal *csp* genes or the "*C. symbiosum*" *csp* gene suppressed the cold sensitivity of BX04. In this regard, it was noteworthy that at 30°C all of the strains were able to form colonies (data not shown), indicating that none of the recombinant plasmids were inherently toxic to the cell. In order to determine if all of the *csp* genes were expressed in BX04, cells were grown in liquid culture at 37°C and induced with 2 mM IPTG, the temperature was decreased to 15°C, and soluble and insoluble fractions were analyzed by SDS-gel electrophoresis. Bands in the soluble fraction consistent with the predicted molecular weights of the Csps were observed for all of the archaeal genes except the two *Halobacterium* sp. strain NRC-1 genes and one of the *H. lacusprofundi* genes (data not shown). A band of the appropriate molecular weight was also apparent for the *M. burtonii* CSD and *M. frigidum* Csp-His (data not shown). All of the proteins visualized were in the soluble fraction, and none appeared to accumulate in the insoluble fraction (data not shown).

The inability of the haloarchaeal *csp* genes to complement in BX04 may indicate that they perform a function(s) in the cell that is not compatible with restoring the *E. coli* growth defect. Alternatively, the haloarchaeal Csps may have a requirement for a higher intracellular salt concentration than is present in *E. coli*. For example, a DNA ligase from *H. volcanii*, LigN, that was purified as a recombinant protein from *E. coli* was inactive in the absence of KCl and displayed increasing activity at KCl concentrations of up to 3.2 M (41).

Structural features predicted to be important for archaeal Csp function in *E. coli*. In order to identify structural features of the proteins that may explain the complementation data, analyses were performed on the amino acid sequences, predicted secondary structures, and tertiary structures generated by homology modeling. In particular, the aromatic and basic residues were examined as they are known to be involved in binding to nucleic acids (25, 51).

The aromatic residues are generally conserved among all of the Csps (Fig. 2), with three of the seven aromatic residues on the nucleic acid binding surface, F12, F20, and F31 (amino acid numbering based on *E. coli* CspA) (16), being identical in all nine sequences. In some positions, the type of aromatic residue varies; e.g., F18 is either phenylalanine or tyrosine. On the nucleic acid binding surface, *E. coli* CspA has five basic and four acidic residues, *M. frigidum* Csp and uncultured *Crenarchaeota* Csp have six basic and five acidic residues, "*C. symbiosum*" Csp has seven basic and three acidic residues, and the halophilic proteins have two basic and seven or eight acidic residues. The ratio of basic to acidic residues is higher in the proteins that complement (*M. frigidum* Csp and uncultured *Crenarchaeota* Csp) compared to those that do not comple-

TABLE 2. Sequence identity between Csps used for complementation analysis

Protein	% Identity										
	E. coli Csp	*M. frigidum* Csp	Uncultured *Crenarchaeota* Csp	"*C. symbiosum*" Csp	*H. marismortui* Csp	*Halobacterium* sp. strain NRC-1 101	*Halobacterium* sp. strain NRC-1 1836	*H. lacusprofundi* 846	*H. lacusprofundi* 1547	*M. burtonii* CSD
E. coli Csp	100	59	39	42	43	36	39	44	41	5.9
M. frigidum Csp		100	36	39	38	32	34	38	36	5.9
Uncultured *Crenarchaeota* Csp			100	73	38	33	36	42	36	6.8
"*C. symbiosum*" Csp				100	40	35	38	42	36	6.4
H. marismortui Csp					100	82	80	91	82	5.1
Halobacterium sp. strain NRC-1 101						100	68	77	71	4.7
Halobacterium sp. strain NRC-1 1836							100	78	90	4.6
H. lacusprofundi 846								100	81	5.1
H. lacusprofundi 1547									100	4.6
M. burtonii CSD										100

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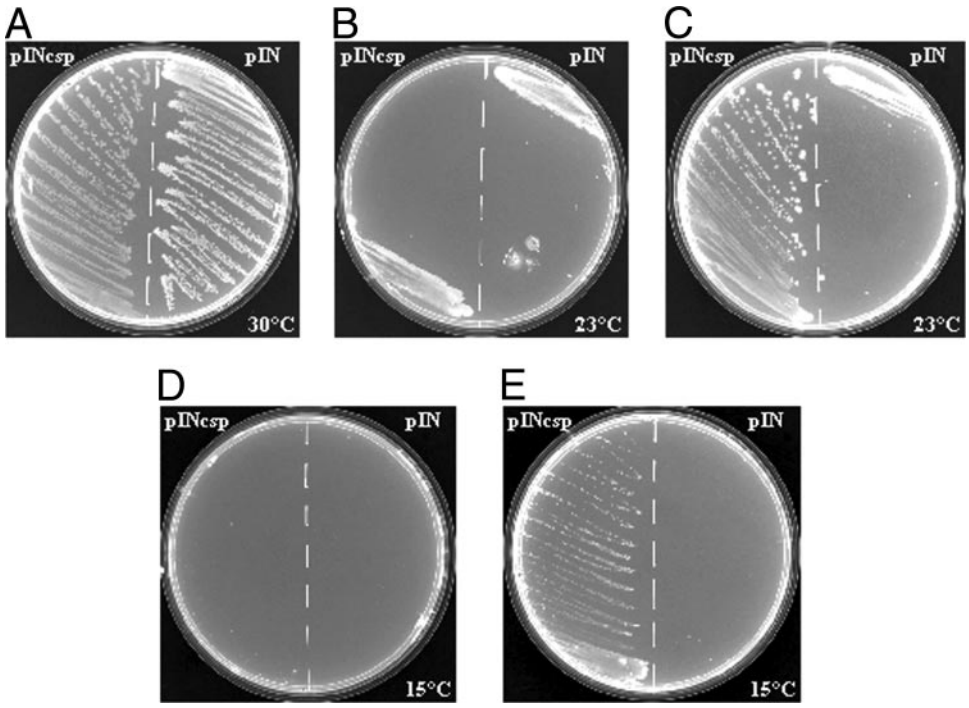

FIG. 1. Complementation of cold sensitivity in *E. coli* BX04 cells expressing *M. frigidum csp*. The pIN-*csp* plasmid and the pIN vector (control) were transformed into cold-sensitive strain BX04 and streaked onto LB plates containing ampicillin with (A, C, E) and without (B, D) 2 mM IPTG, and the plates were incubated at 30°C (A), 23°C (B, C), or 15°C (D, E).

ment ("*C. symbiosum*" Csp and the haloarchaeal proteins), with the ratio being especially low in the halophilic proteins. These data indicate that the main differences in the Csp sequences relate to the content and distribution of positively charged amino acids. It is noteworthy that the high content of acidic residues in the Csps from halophiles is consistent with the general overrepresentation of acidic residues as an adaptation to growth in highly saline environments (28).

Secondary-structure predictions identified five β-strands in all of the Csps except that of *Halobacterium* sp. strain NRC-1 (encoded by gene 101), which had an additional β-strand predicted at the C-terminal end of the protein (data not shown). No three-dimensional structures are available for archaeal Csps. However, the three-dimensional structure of *E. coli* CspA has been resolved by X-ray crystallography (46) and nuclear magnetic resonance spectroscopy (16, 38). The nucleic acid binding surface is composed of residues 9 to 13 (on β1), 14 to 17 (on L1), 18 to 21 (on β2), 30 to 33 (on β3), 34 to 49 (on L3), and 58 to 61 (on L4) (16). The homology models of the eight archaeal Csps displayed the characteristic β-barrel structure, with β1, β2, and β3 strands opposing the β4 and β5 strands (Fig. 3). *E. coli* CspA contains eight basic residues, seven of which are accessible, and of these seven, four are located on the nucleic acid binding side of the barrel (Fig. 3A, strands β1 to β3 and loops L1, L3, and L4). *M. frigidum* Csp also contains eight basic amino acids, four of which are exposed on the binding side of the barrel (Fig. 3B). The two Csps from the *Crenarchaeota* have even higher numbers of positive residues (12 and 11), but only 4 of them appear accessibly located on the binding surface of the barrel (Fig. 3C and D).

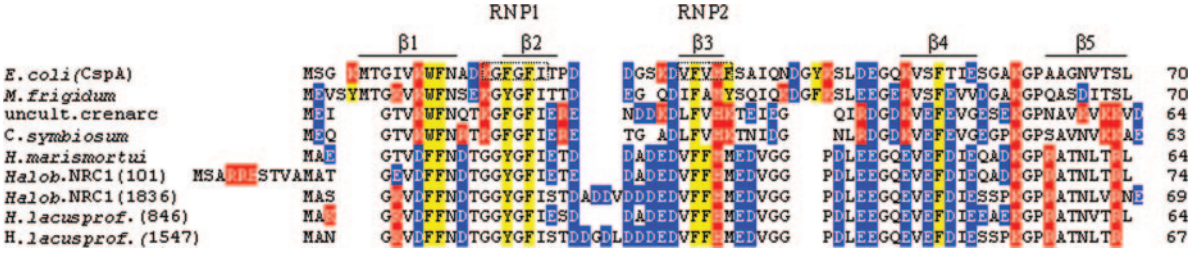

FIG. 2. Primary-structure analysis of Csp homologs. Amino acid sequence alignments of Csp homologs from *E. coli* (CspA), *M. frigidum*, uncultured *Crenarchaeota*, "*C. symbiosum*," *H. marismortui*, *Halobacterium* sp. strain NRC-1 (genes 101 and 1836), and *H. lacusprofundi* (genes 846 and 1547). Aromatic residues, negatively charged residues, and positively charged residues are highlighted in yellow, blue, and red, respectively. β-Strands in *E. coli* CspA are indicated by bars, and the residues composing the RNA binding motifs, RNP1 and RNP2, are boxed. The lengths of the proteins are shown on the right in amino acids.

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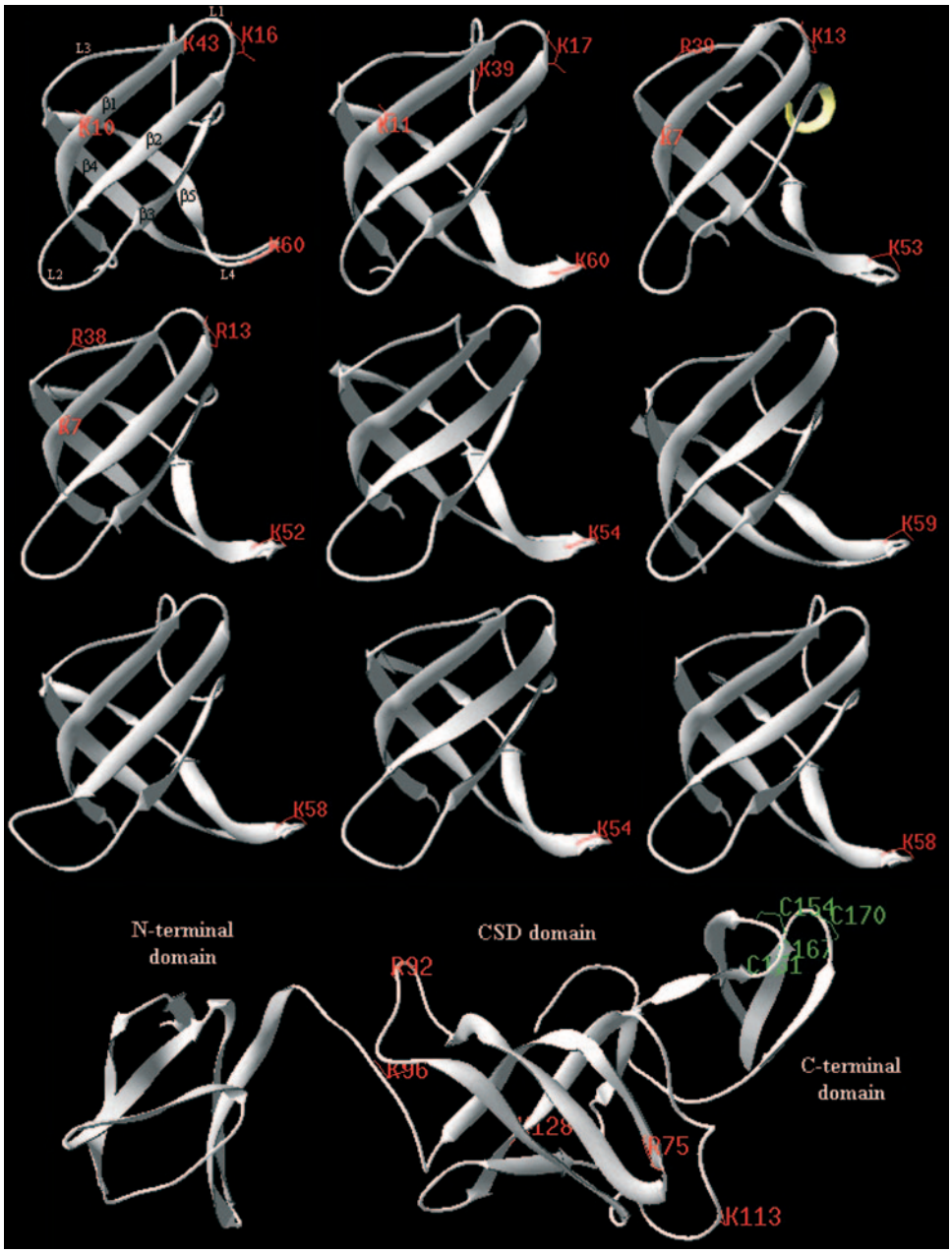

FIG. 3. Three-dimensional structure models of Csp homologs. The nuclear magnetic resonance structure of *E. coli* CspA (A) and homology models of *M. frigidum* Csp (B), uncultured *Crenarchaeota* Csp (C), "*C. symbiosum*" Csp (D), and Csps from *H. marismortui* (E), *Halobacterium* sp. strain NRC-1 (genes 101 and 1836) (F and G), *H. lacusprofundi* (genes 846 and 1547) (H and I), and *M. burtonii* CSD (J) are shown. Positively charged residues exposed on the surface are in red writing. *E. coli* Csp β-sheets 1 to 5 are in black writing. *E. coli* Csp loops 1 to 4 are in white writing. *M. burtonii* CSD cysteine residues typical of Zn ribbons are in green writing. The models are presented alphabetically from left to right, with A in the top left corner and J at the bottom.

The Csp homologs from the haloarchaea possess between four and six basic residues, only two or three of which are accessible, and only one of these is located on the β1-to-β3 surface (Fig. 3E to I).

The homology model of the *M. burtonii* CSD has three domains (Fig. 3K). The central domain consists of five strands organized in a β-barrel structure very similar to that of the Csps. This domain contains 11 basic amino acids, 5 of which are exposed on the surface that corresponds to the binding surface of Csps.

The four proteins that were able to suppress the cold sensitivity of *E. coli* BX04 exhibited the highest number of accessible positive charges on the binding surface of their barrel structures (Table 3). Residues K10, K16, and K60 are located on β1, L1, and L4, respectively, and are conserved in all three Csp homologs. The remaining solvent-exposed residues of *E.*

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TABLE 3. Tertiary-structure characteristics of Csp homologs

Protein or gene	Total no. of basic residues	No. of accessible basic residues exposed on β1, β2, β3, L1, L3, and L4[a]
E. coli CspA	8	4
M. frigidum Csp	8	4
Uncultured *Crenarchaeota* Csp	12	4
M. burtonii CSD	11	5
"*C. symbiosum*" Csp	11	4
H. marismortui Csp	4	1
Halobacterium sp. strain NRC-1 gene 101[b]	4	1
Halobacterium sp. strain NRC-1 gene 1836	5	1
H. lacusprofundi gene 846	6	1
H. lacusprofundi gene 1547	5	1

[a] Content of basic amino acid residues and of accessible basic residues exposed on strands β1 to β3 and loops L1, L3, and L4 is shown. All data are based on homology modeling (Swiss-PdbViewer).

[b] The protein encoded by this gene contains a total of seven basic residues (see Fig. 2). The homology modeling did not include the first five residues.

coli CspA, *M. frigidum* Csp, and uncultured *Crenarchaeota* Csp belong to loop L3. These common structural features may be involved in the binding of *E. coli* nucleic acid.

Biochemical analysis of *M. frigidum* Csp. *M. frigidum* Csp was purified as a recombinant protein free of an affinity tag with the cleavable intein-chitin binding system (see Materials and Methods). Following expression in *E. coli* BL21(DE3), 5 mg liter^{-1} pure *M. frigidum* Csp was yielded (determined by Bradford assay and amino acid analysis) (Fig. 4). The molecular mass of the purified protein determined by MALDI-TOF MS was 7,814.6 Da (data not shown), consistent with the theoretical mass of 7,815.6 Da.

The folding kinetics of *M. frigidum* Csp were assessed by monitoring its transition curve in the presence of a transverse urea gradient as previously described (10, 53). Unfolding (N⇆U) and refolding (U⇆N) transitions of *M. frigidum* Csp were both examined by TUG-GE (Fig. 5). Multiple bands indicative of protein variants were revealed. The low-intensity upper bands arose from a translation product that was initiated from an internal methionine (residue 6) that produced an unfolded protein (data not shown). This was determined by overexpressing and purifying an *M. frigidum* Csp hybrid that initiated translation at Met6, visualizing the product by TUG-GE, and analyzing the molecular masses of all of the bands produced by the full-length and hybrid *M. frigidum* Csps from TUG gels (L. Giaquinto and R. Cavicchioli, unpublished results).

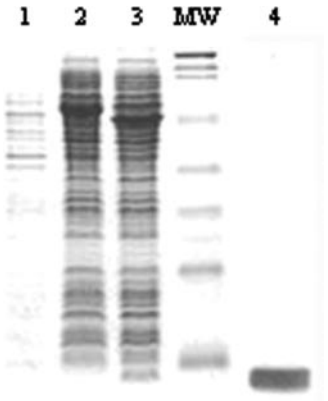

FIG. 4. Expression and purification of *M. frigidum* Csp. SDS-gel electrophoresis analysis of the purification of *M. frigidum* Csp by affinity chromatography on chitin beads. *M. frigidum* Csp was purified from crude lysate of BL21(DE3) cells expressing the *M. frigidum* Csp-intein-CBD fusion. Shown are the insoluble fraction (lane 1), the soluble fraction in sample buffer without DTT (lane 2), the soluble fraction in sample buffer with 70 mM DTT (lane 3), the purified *M. frigidum* Csp (lane 4), and a broad-range protein molecular size standard (lane MW; Bio-Rad) corresponding to 224, 122, 90, 51.5, 35.3, 28.7, 21, and 7.2 kDa.

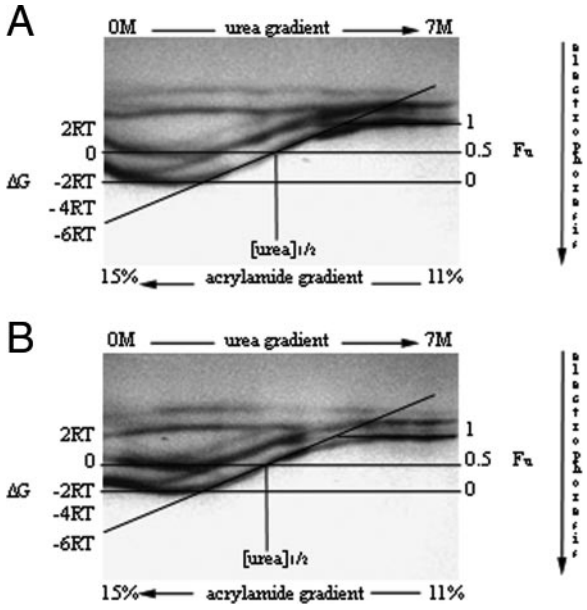

FIG. 5. TUG-GE of *M. frigidum* Csp. Unfolding (N⇆U) (A) and refolding (U⇆N) (B) transition curves of *M. frigidum* Csp in a TUG (0 to 7 M) perpendicular to the direction of electrophoresis is shown. The urea gradient is counterbalanced by an inverse acrylamide gradient (15 to 11%). The free energy of unfolding at 0 M urea was calculated by graphically extrapolating from the transition region, and the concentration of urea at $\Delta G = 0$ is $[\text{urea}]_{1/2}$. F_u, fraction of unfolded molecules; ΔG, free-energy difference between folded and unfolded states; $[\text{urea}]_{1/2}$, concentration of urea at equilibrium; R, universal gas constant (8.314 kJ mol^{-1}); T, absolute temperature.

For the high-intensity lower bands that correspond to the full-length *M. frigidum* Csp, a single transition was observed between the initial (0 M urea) and final (7 M urea) states. Identical transition profiles were observed for the N⇆U and U⇆N processes. These results demonstrate that *M. frigidum* Csp is a single-domain protein that folds by a reversible two-state mechanism. The presence of two bands at high urea concentrations (when the protein is fully unfolded) is indicative of the presence of two structural variants of the full-length *M. frigidum* Csp. These variants may arise as a result of post-translational modification of the *M. frigidum* Csp in *E. coli*.

To assess the conformational stability of *M. frigidum* Csp, ΔG and $[\text{urea}]_{1/2}$ were calculated at 7°C for the bands corresponding to the full-length *M. frigidum* Csp (Fig. 5). The values

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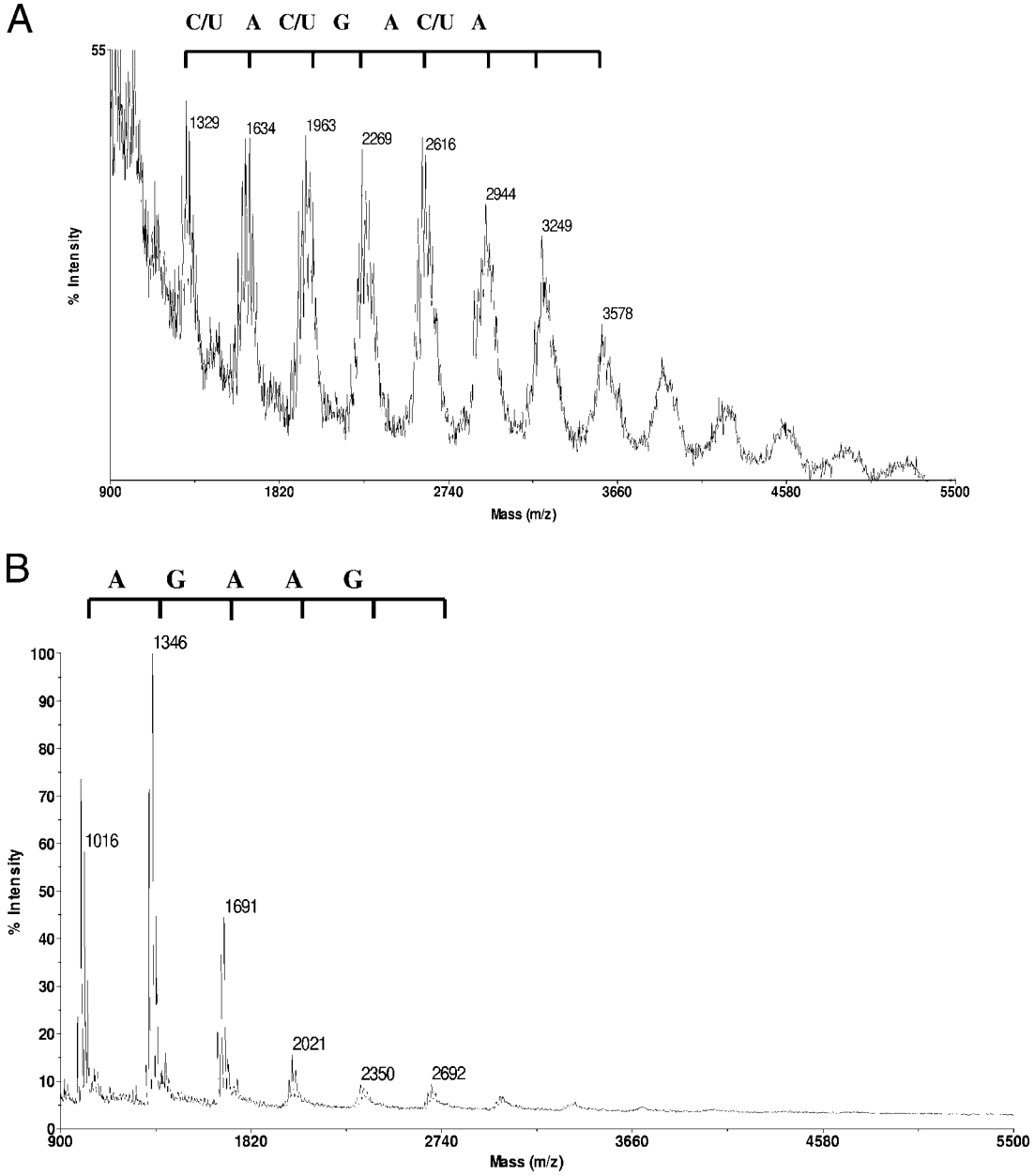

FIG. 6. Mass spectra of *M. frigidum* Csp. Shown are the positive-ion MALDI-TOF mass spectra of *M. frigidum* Csp incubated in subtilisin (A) and *M. frigidum* Csp incubated in subtilisin and RNase A (B) determined by C_{18} ZipTip extraction and a 3-HPA matrix. The ribonucleotide bases corresponding to the mass difference between consecutive peaks are indicated. The average residue mass for each of the ribonucleotide bases is as follows: adenosine, 329.21; cytidine, 305.18; uridine, 306.17; guanosine, 345.21.

calculated were as follows: ΔG, 11.4 kJ mol^{-1}; $[\text{urea}]_{1/2}$, 3.2 M. The folding state of *M. frigidum* Csp was further assessed by monitoring changes in its secondary structure in the far-UV region (180 to 250 nm) by CD spectroscopy, and after thermal denaturation at 60°C, *M. frigidum* Csp exhibited characteristics typical of nonnative molecules (data not shown) (47). These properties are consistent with the protein being from a psychrophile and being thermolabile.

The UV absorption spectrum of *M. frigidum* Csp showed a maximum at 260 nm and an A_{280}/A_{260} ratio of 0.77 (data not shown). The strong signal at 260 nm is likely to indicate the presence of nucleic acid in the protein sample. To determine if *E. coli* RNA was bound by *M. frigidum* Csp, the protein was analyzed by MALDI-TOF MS with a 3-HPA matrix. Initial analysis in the absence of subtilisin (data not shown) revealed a few peaks of very weak signal intensity in the 900- to 5,500-Da mass range. However, when an analysis was performed with *M. frigidum* Csp samples to which either subtilisin (Fig. 6A) or both subtilisin and an RNase inhibitor (data not shown) had been added, the spectra showed a systematic progression of peaks differing by the mass of ribonucleotide residue mass units. In particular, a mass difference of 347 Da, close

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to 345 Da was observed, consistent with the residue mass of guanosine. This is 16 Da higher in mass than the residue mass of deoxyguanosine and indicates that the series corresponds to RNA rather than DNA. The MALDI-TOF spectrum of *M. frigidum* Csp hydrolyzed with subtilisin in the presence of an RNase inhibitor (data not shown) produced a similar mass distribution, indicating that it was unlikely that RNases were active during sample handling for MALDI. The combined addition of subtilisin and RNase A to *M. frigidum* Csp produced MALDI signals with a higher intensity and in a lower mass range (Fig. 6B), indicating that RNase A was able to more extensively hydrolyze the target RNA when the *M. frigidum* Csp was removed by subtilisin hydrolysis. This suggests that an in-solution association between *M. frigidum* Csp and RNA not only survives the extraction-and-purification procedure but also offers protection from RNase degradation in the vicinity of the binding site. The series of peaks observed in the MALDI-TOF spectra most probably reflect variable endogenous degradation of the exposed RNA end. Since RNase A is specific for single-stranded RNA, our data indicate that single-stranded RNA was specifically associated with *M. frigidum* Csp sample preparation.

Collectively, these data may indicate that an *M. frigidum* Csp-RNA complex protects the nucleic acid from degradation, since (i) the RNA series is easily observed only when the protein is removed by subtilisin hydrolysis and (ii) more extensive hydrolysis of the RNA is observed when RNase A is added in addition to subtilisin.

DISCUSSION

The complementation studies show that archaeal Csp and CSD fold proteins representing both the *Euryarchaeota* (*M. frigidum* and *M. burtonii*) and *Crenarchaeota* (uncultured marine picoplankton) can function effectively to rescue a growth defect in the bacterium *E. coli*. This illustrates that some archaeal Csp and CSD fold proteins have retained a high degree of functional similarity to their bacterial counterparts throughout evolution in the *Archaea*. The three genes that complemented cold sensitivity were from three archaea that inhabit cold environments (an Antarctic lake and the deep sea), indicating that this class of genes plays a particular role in cold adaptation in archaea. The lack of *csp* homologs in archaeal thermophiles and hyperthermophiles lends further support to this. Our understanding of the general characteristics of cold-adapted archaea is limited (8) and can now be expanded to include a role for Csp and CSD fold proteins.

Characteristics of archaeal *csp* and *CSD* complementation. *M. frigidum csp* complemented BX04 in the presence of IPTG (Fig. 1), whereas uncultured *Crenarchaeota csp* was able to complement the growth defect with and without IPTG (data not shown). This is consistent with a previous study with *E. coli csp* genes which found that *cspA*, *cspE*, and *cspF* were able to complement only in the presence of IPTG and *cspG* and *cspI* were able to do so with and without IPTG (63). The level of amino acid sequence similarity does not explain our complementation results, as the uncultured *Crenarchaeota* Csp has lower primary sequence identity to *E. coli* CspA (39%) than *M. frigidum* Csp has to *E. coli* CspA (59%) (Table 2). However, the three proteins able to complement the growth defect of BX04, *M. frigidum* Csp, uncultured *Crenarchaeota* Csp, and *M. burtonii* CSD, do have particular structural features identifiable in their modeled tertiary structures in common with *E. coli* CspA that are not features of the other archaeal Csps. In *E. coli* CspA (25) and *Bacillus subtilis* CspB (51), a cluster of aromatic or basic amino acids on the β-barrel surface plays a key role in the interaction of the protein with nucleic acids. Positive charges attract the negatively charged nucleic acid by electrostatic interaction, and the aromatic patches bind and melt nucleic acid secondary structure to facilitate transcription and translation at low temperatures (40). In the archaeal Csps, while the aromatic cluster of residues is largely conserved in all of the proteins, the number and location of the acidic and basic residues vary among the Csp homologs and the *M. burtonii* CSD. The *M. frigidum* Csp, uncultured *Crenarchaeota* Csp, and *M. burtonii* CSD have a higher content of solvent-exposed basic residues (Table 3), and these are located on the nucleic acid binding surface of their β-barrel structures (Fig. 3B, C, and J), similar to the number and location of solvent-exposed basic residues for *E. coli* CspA (Fig. 3A). These structural features are the main characteristics that can be identified that explain the complementation results.

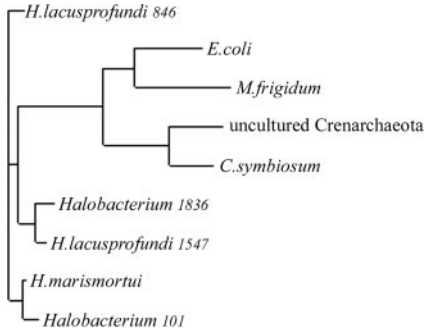

FIG. 7. Phylogenetic tree of Csp homologs. The tree was constructed with the PHYLIP server.

The phylogenetic relationship of the archaeal Csp homologs with *E. coli* CspA reveals that *E. coli* CspA, the *M. frigidum* Csp, and the two *Crenarchaeota* Csps form a cluster distinct from the other Csps (Fig. 7). The "*C. symbiosum*" Csp clusters with the complementing genes. As the "*C. symbiosum*" Csp also has four accessible positive charges predicted on the binding surface of the protein (Fig. 3 and Table 3), it may be expected to complement BX04. However, the ratio of basic to acidic residues on the nucleic acid binding surface is considerably higher for the "*C. symbiosum*" Csp (7:3) compared to the *M. frigidum* Csp and the uncultured *Crenarchaeota* Csp (6:5) and the *E. coli* Csp (5:4), and this charge balance for the "*C. symbiosum*" Csp may adversely affect its interactions with nucleic acid targets involved in complementation. We also noted that although it is not apparent in the *E. coli* Csp three-dimensional structure, between strands β3 and β4, a short α-helix region was predicted in the secondary-structure plot of the *E. coli* Csp, *M. frigidum* Csp, and uncultured *Crenarchaeota* Csp that is not predicted in the "*C. symbiosum*" Csp (data not shown).

***M. burtonii* CSD.** In addition to the cold sensitivity of the *E. coli* quadruple-deletion mutant BX04 (63), the deletion of two

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of the three *csp* genes in *B. subtilis* causes a severe reduction in growth ability (23). The growth defects can be complemented by overexpressing a specific set of proteins that includes homologous Csps, or the S1 domain of PNPase in *E. coli*, or initiation factor IF1 in *B. subtilis*. The primary sequences of both the S1 domain and IF1 have little similarity to those of Csps, but their three-dimensional structures are very similar and functional overlap between these proteins has been proposed (59, 63). The *M. burtonii* CSD is the first example of an archaeal protein that fulfills an functional role analogous to that of the *E. coli* S1 domain and the *B. subtilis* IF1 domain.

The *M. burtonii* CSD is one of two *M. burtonii* CSD proteins originally identified in a comparative genomic analysis as hypothetical proteins with structural features similar to those of Csps (45). Subsequent proteomic analysis found that both proteins were synthesized in cells growing at 4°C, thereby demonstrating that they are not only functional but important for low-temperature growth (21). Both proteins are encoded within a region of archaeal genomes that was previously described as a superoperon involved in RNA and protein processing, with their predicted RNA binding fold lending support for their role in RNA processing in *M. burtonii* (21, 30). The C-terminal domain of *M. burtonii* CSD has characteristics of Zn ribbon proteins, containing two pairs of closely spaced cysteine residues separated by beta strands (Fig. 3J). Archaeal proteins that contain Zn ribbons are likely to be involved in nucleic acid binding and particularly transcription (1), providing further support for a putative role in RNA processing. The present study extends our knowledge of the *M. burtonii* proteins from the superoperon by demonstrating that the *M. burtonii* CSD can complement *csp* deletions that cause cold sensitivity in *E. coli*.

The *M. frigidum* Csp. Biochemical analysis of the *M. frigidum* Csp demonstrate that it is a single-domain protein that folds by a reversible two-state mechanism (Fig. 5), consistent with the folding kinetics described for bacterial Csp homologs (33, 39, 43, 47, 48, 49). The ΔG of 11.36 kJ mol^{-1} and the $[\text{urea}]_{1/2}$ of 3.2 M calculated at 7°C are lower than the ΔG and $[\text{urea}]_{1/2}$ values of Csp homologs from *E. coli* (43) and *B. subtilis* (50) that were calculated by fluorescence at pH 7 and 25°C. The lower conformational stability of the *M. frigidum* Csp is likely to reflect the increased flexibility and decreased stability of the protein, consistent with adaptations typical of cold-adapted proteins (52).

The ability of *M. frigidum csp* to complement function in vivo is consistent with the ability of the *M. frigidum* Csp to bind *E. coli* RNA. Evidence for the latter came from UV absorption spectroscopy and MALDI-TOF MS analyses (Fig. 6). Moreover, the specificity of RNase A for single-stranded RNA indicates that *M. frigidum* Csp binds single-stranded RNA. The *M. frigidum* Csp-RNA association appears to offer some protection from RNase degradation, since the RNA series is observed only when the protein is hydrolyzed by subtilisin, and removal of the protein by subtilisin hydrolysis allows more extensive degradation of the RNA by RNase A (Fig. 6).

M. frigidum is incapable of growth above ~18°C and has proven difficult to culture in the laboratory, and few studies examining the organism's growth properties or probing mechanisms of adaptation have been reported (17, 36). The ability to perform heterologous protein studies provides opportunities for examining the molecular characteristics that distinguish cold-adapted archaea that possess a restricted growth temperature range (stenopsychrophiles, e.g., *M. frigidum*) from eurypsychrophilic archaea (e.g., *M. burtonii*) that can tolerate a wide range of temperatures extending into the mesophilic range (8), a question about thermal adaptation that is of broad relevance to all microorganisms that live in cold environments (15).

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