Conserved TRAM Domain Functions as an Archaeal Cold Shock Protein via RNA Chaperone Activity

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Cold shock proteins (Csps) enable organisms to acclimate to and survive in cold environments and the bacterial CspA family exerts the cold protection via its RNA chaperone activity. However, most Archaea do not contain orthologs to the bacterial csp. TRAM, a conserved domain among RNA modification proteins ubiquitously distributed in organisms, occurs as an individual protein in most archaeal phyla and has a structural similarity to Csp proteins, yet its biological functions remain unknown.

Through physiological and biochemical studies on four TRAM proteins from a cold adaptive archaeon Methanolobus psychrophilus R15, this work demonstrated that TRAM is an archaeal Csp and exhibits RNA chaperone activity. Three TRAM encoding genes (Mpsy_0643, Mpsy_3043, and Mpsy_3066) exhibited remarkable cold-shock induced transcription and were preferentially translated at lower temperature (18°C), while the fourth (Mpsy_2002) was constitutively expressed. They were all able to complement the cspABGE mutant of Escherichia coli BX04 that does not grow in cold temperatures and showed transcriptional antitermination. TRAM3066 (gene product of Mpsy_3066) and TRAM2002 (gene product of Mpsy_2002) displayed sequence-non-specific RNA but not DNA binding activity, and TRAM3066 assisted RNases in degradation of structured RNA, thus validating the RNA chaperone activity of TRAMs. Given the chaperone activity, TRAM is predicted to function beyond a Csp.

Keywords: archaea, TRAM, RNA chaperone, cold shock protein, transcriptional antitermination, RNA binding, assist structure RNA degradation

INTRODUCTION

Cold shock proteins (Csps) are essential for organisms to grow and survive in cold environments. These proteins enable organisms to acclimate to downshifts in temperature by destabilization of cold-induced misfolded RNAs that may cause premature transcription termination, inefficient RNA turnover, and reduced translation. Two categories of bacterial Csps are classified in terms of their working mechanisms, the cold shock protein A (CspA) family of proteins functions mainly as RNA chaperones by affecting transcription or translation of a nascent transcript (Jiang et al., 1997), while the DEAD-box RNA helicases and exoribonuclease unwind and degrade misfolded RNAs under cold temperatures. Unlike RNA helicase, RNA chaperones such as the CspA family of Csps remodel RNAs without a requirement for ATP input (Semrad, 2011). Escherichia coli contains nine Csp proteins, among which CspA, CspB, CspG, and CspE are essential for cold acclimation of the bacterium. Typically, the cspA gene does not express at mesophilic temperatures but is remarkably
induced by cold shock, and the level is reduced after the organism acclimates to the cold (Jiang et al., 1997). Differently, the CspS in *Bacillus* are retained after cold shock (Graumann et al., 1997). *Caulobacter crescentus* also has four CspS: CspA and CspB are cold-induced, and CspC and CspD are induced only in stationary phase (Mazzon et al., 2012). This indicates that the bacterial CspS exert biological roles far beyond cold protection.

The most intensively studied CspA family are basic small proteins of approximately 70 amino acid residues, and the native folds contain an OB (oligonucleotide binding)-fold for binding RNA (Jiang et al., 1997). They accumulate in *E. coli* cells when encountering temperature downshifts and assist the bacterium in acclimation to cold. Through RNA chaperone activity, the CspA family proteins prevent or melt incorrect RNA duplexes formed under lower temperatures, which would hinder efficient translation and regular turnover of a transcript.

Bacterial CspS regulate gene expression at posttranscriptional level. Deletion of cspE is shown to affect the transcription of genes containing promoter-proximal elements that induce premature transcription termination. *E. coli* CspC and CspE stabilize the RpoS transcript, a sigma factor that specifically exerts roles under adverse conditions (Shenhar et al., 2012).

However, no Csp has yet been reported for Archaea, although they are abundant in permanently cold environments, including the earth poles and permafrost (Shcherbakova et al., 2016). With the exception of a few psychrophiles and mesophiles (Giaquinto et al., 2007), most Archaea do not contain orthologs to the bacterial *csp*. Transcriptomic analyses of the cold-adaptive methanogenic Archaea *Methanolobus psychrophilus* R15 and *Methanococcoides burtonii* have found that genes encoding small proteins of approximately 70 amino acids that possess a TRAM domain were strongly induced at lower temperatures (Campanaro et al., 2011; Chen et al., 2012). These results suggested that TRAM domain proteins might be involved in the cold adaptation of Archaea. Although there is no sequence homology between TRAM proteins and CspS, they possess the same properties in protein size of approximately 7 kDa, with positively charged amino acids surrounding a surface-exposed aromatic patch, and similar conformation of several antiparallel β-strands containing two RNA-binding motifs. In a recent work, a small protein possessing TRAM domain from a cold-adaptive methanogenic archaeon *Methanococcoides burtonii* was determined to preferentially bind tRNAs and 5S rRNA in vitro (Taha et al., 2016), implying its RNA chaperone activity.

**MATERIALS AND METHODS**

**Microbial Strains and Culture Conditions**

Strains used in this study and their characteristics are listed in Supplementary Table S1. *E. coli* BX04 and plasmid pINIII were kindly provided by Professor Masayori Inouye at the University of Medicine and Dentistry of New Jersey. *E. coli* RL211 was kindly provided by Professor Robert Landick at the University of Wisconsin-Madison. The strains were routinely grown at 37°C in Luria-Bertani (LB) broth.

*Methanobacterium psychrophilus* R15 is preserved in our laboratory and cultured at 18°C in mineral medium containing 20 mM trimethylamine, as previously described (Zhang et al., 2008). Growth was monitored by determining the optical density at 600 nm (OD600). For cold shock treatment, the mid-log-phase cultures (OD600 0.41 to 0.43) were pregrown at 18°C or 30°C and incubated at 4°C for 0.5, 1.0, 2.0, and 4.0 h.

**RNA Extraction and qPCR Assay**

Three biological replicates of the cold-shocked culture were collected. Five milliliters of each culture was used for RNA extraction using TRIZol as previously described (Li et al., 2015). The extracted RNA was quantified using the NanoDrop ND-100 Spectrophotometer (Gene Company limited, Hong Kong) and analyzed with a 1% agarose gel.

To completely remove the contaminant genomic DNA, 2 µg of each RNA sample was treated with two units of DNase I (Promega, Madison, WI, United States) at 37°C for 5 h. RNA-dependent DNA synthesis was performed using the Moloney murine leukemia virus reverse transcriptase (MMLV RT, Promega) according to the manufacturer’s protocol with random primers (Promega) and 2 µg of DNA-free RNA as the template. The generated cDNA was used as the template for quantitative PCR (qPCR). Real-time qPCR was conducted using the Eppendorf Mastercycler system (Eppendorf, Hamburg, Germany) with 25 µL of SYBR green Premix (TaKaRa) and corresponding primers listed in Supplementary Table S2. The PCR thermal cycle was 95°C for 30 s, followed by 40 cycles of 95°C for 10 s, annealing for 30 s and 72°C for 30 s. Different annealing temperatures were used for the four TRAM genes: 51°C for *Mpsy_0643*, 53°C for *Mpsy_3043*, 59.8°C for *Mpsy_3043*, and 54.3°C for *Mpsy_3066*. Transcript quantification was determined using the comparative threshold cycle (CT) values. To estimate the copy numbers of the transcripts, the expression plasmids pIN-0643, pIN-2002, pIN-3043, and
pIN-3066, constructed in the following section, were serially diluted to generate a calibration curve by PCR amplification using the corresponding primer pairs (Supplementary Table S2). The 16S RNA gene copies were used as a biomass reference. By using the primer pair P3/P4 listed in Supplementary Table S2, almost complete 16S rDNA sequence was amplified and the product was applied as qPCR template to quantify the copy numbers of 16S rRNA against the 16S rRNA gene copies and shown as per 10−4 curve. Quantification of a gene copy number was normalized by centrifugation, resuspended in 30 μl of lysis buffer (pH 8.0) containing 20 mM Tris-HCl, 1 M NaCl and 10% glycerol and lysed by ultrasonication at 300 W using a cycle of 3 s sonication and 3 s pause for 30 min on ice. After cell debris was removed by centrifugation, the supernatant was mixed with 0.5% (V/V) polyethyleneimine to remove any contaminated nucleic acids. The mixture was centrifuged at 10,000 g for 30 min at the same temperature, and proteins in the supernatant were collected by precipitation with (NH₄)₂SO₄ powder to a final 70% saturation. The protein precipitation was dissolved in 15–20 μl of binding buffer (20 mM Tris-HCl, 100 mM NaCl, and 20 mM imidazole, pH 8.0) and dialyzed overnight at 4°C against the same buffer in 3.5 kDa-pore size dialysis tubing (Spectrumlabs, United States). The dialyzed mixture was centrifuged, and the supernatant was passed through a 0.22-μm filter. The His6-tagged recombinant proteins were purified by passage on a HisTrap HP affinity column (GE Healthcare), followed by HiTrap Q HP anion exchange column (GE Healthcare) and gel filtration chromatography through a Hilo 16/600 Superdex 75 pg column (GE Healthcare). Proteins were concentrated with Amicon Ultra-15 concentrators (Millipore) when necessary. The purified proteins were examined by Tricine-SDS-PAGE, and the concentrations were determined using a bicinchoninic acid (BCA) protein concentration assay kit (Pierce, Thermo scientific). The purity of proteins (>99%) and residual nucleic acid were confirmed using a NanoDrop ND-100 Spectrophotometer. To identify the purified proteins, they were digested with trypsin in gels according to the method of Shevchenko et al. (2006) and analyzed by AB Sciex 5800 MALDI-TOF/TOF mass spectrometer.

**Western Blot**

A total of 25 ml of each cold-shocked R15 culture, which was cultured for different durations, was harvested at 12,000 g for 3 min, resuspended in 400 μl PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, pH 7.4), and lysed by ultrasonication at 240 W in a cycle of 3 s sonication and 3 s pause for 15 min on ice. All manipulations were conducted following standard protocols (Sambrook and Russell, 2001) with a few modifications. Total cellular protein was electrophoresed on Tricine-SDS-PAGE below 100 mA for 1.5 h and transferred to a 0.1-μm nitrocellulose filter membrane (Easybio, Beijing, China) under 100 V for 1 h. To fix the proteins to the membrane surface, ddH₂O and 1 × TBS (2.42 g Tris, 8.0 g NaCl in 1 L, pH 7.6) were used to wash the membrane with gentle shaking for 5 min, and 0.2% glutaraldehyde diluted in 1 × TBS was used to wash the membrane for 45 min. The TRAM3066-specific peptide and TRM2002 total protein-generated antibodies were prepared by the MBL (Beijing Biotech Co., Ltd.). The secondary antibody was purchased from Easybio (Beijing, China).

The density of the Western blotting protein band was determined using Bio-Rad Quantity One (Bio-Rad, Hercules, CA, United States), and the amount of protein was calculated by referring to the gray value of the overexpressed protein.
RNA Probe Preparation

The Pentaprobe (PP) library developed by Bendak et al. (2012) that comprises twelve 100-bp oligonucleotides encompassing 1,024 possible 5-nucleotide sequence combinations was synthesized by Sangon (Shanghai, China). They were inserted into pUC57 at BamHI/ApaI sites downstream of the T7 promoter, and then used as templates for in vitro transcription using the MEGA shortscript T7 Kit (Ambion, Thermo Fisher Scientific) and primer pairs (P35–P47) listed in Supplementary Table S2. The in vitro transcription products were purified on 7% urea-PAGE using a ZR small-RNA PAGE Recovery Kit (Zymo Research), and the concentrations were determined using NanoDrop ND-100 Spectrophotometer. All in vitro transcribed RNAs were 3′-end biotinylated using a 3′-End Biotinylation Kit (Pierce, Thermo Scientific).

RNA Electrophoretic Mobility Shift Assay (rEMSA)

An RNA binding assay was performed in a 20-µl reaction mixture containing binding buffer (20 mM Tris-HCl, pH 8.0, 5 mM MgCl₂, 10 mM KCl, 1 mM DTT, and 10% of glycerol), biotin-labeled RNA substrate, and purified recombinant proteins. After incubation at 25°C for 15 min, the reaction mixtures were loaded onto a 6% polyacrylamide gel and electrophoresed under 100 V for 1 h in 0.5 × TBE running buffer (1 mM EDTA, 45 mM Tris-boric acid, pH 8.0). Free RNA and RNA-protein complexes in gels were transferred to a nylon membrane at 380 mA for 45 min using a Trans-Blot transfer (Bio-Rad Laboratories, Hercules, CA, United States). After cross-linking using a GS Gene Linker™ UV Chamber (Bio-Rad Laboratories, Hercules, CA, United States) for 2 min, a Chemiluminescent Nucleic Acid Detection Module kit (Thermo Scientific) was used to detect chemiluminescence by exposure on a Tanon-5200 Multi instrument (Tanon Science & Technology Co. Ltd., Shanghai, China).

Surface Plasmon Resonance (SPR) Assay

A surface plasmon resonance (SPR) assay was performed to determine the binding affinities of TRAM3066 and TRAM2002 to ssRNAs on a BIACore 3000 system (Uppsala, Sweden) as described previously (Li et al., 2010) with minor modifications. The Pentaprobe DNAs with an A₁₈C₃ tail were PCR-amplified by primer pair of P50/P51 in Supplementary Table S2 and then used as the transcription templates for Pentaprobe RNA substrates used in SPR assay. A streptavidin-coated sensor chip SA (Sensor-Chip SA®, GE Healthcare) was first conditioned with 3–5 injections (10 µl/min) of 1 M NaCl in 50 mM NaOH until a stable baseline was obtained. The chip cells were washed twice with 0.05% SDS in buffer I (10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 3 mM EDTA, 0.005% Tween20) for 3 min. The biotinylated single-stranded oligonucleotides (SPR linker) were diluted to 10 µM in buffer II (10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 1 mM EDTA) and immobilized on a streptavidin-coated sensor chip SA at a flow rate of 5 µl/min for 10 min. Fifty µM NaOH was injected at 5 µl/min to remove unbound SPR linker until the response units (RU) reached 150. The A₁₈C₃ probe and Pentaprobe RNAs containing A₁₈C₃ tail were immobilized to SPR linkers in different flow cells by manual injection at a flow rate of 1 µl/min until the RU reached a stable state. TRAMs were diluted to 0 to 100 µM by a twofold dilution series with buffer I and injected using the K-inject command at room temperature. CspA and BSA were included as positive and negative controls, respectively. Buffer I (30 µl/min) was used as the running buffer. At the end of each cycle, chip cells were washed with 0.1% SDS to remove bound protein. Data analysis was performed using the BIA evaluation 3.0 program (BIACore AB, Uppsala, Sweden).

In Vivo Transcription Antitermination Assay

To determine the in vivo nucleic acid melting activity of TRAMs, each of Mpsy_0643, Mpsy_2002, Mpsy_3043, and Mpsy_3066 cloned pINIII plasmids was transformed into E. coli RL211. The transformants were cultured overnight in LB complemented with 100 µg/ml ampicillin and diluted 100-fold in fresh medium. When the culture grew to an OD600 of 1.0, 6 µl cultures were spotted onto LB plates containing 100 µg/ml ampicillin, with or without 0.2 mM IPTG and 30 µg/ml chloramphenicol, and grown for 2–3 days. E. coli cspE and pINIII were used as positive and negative controls, respectively. Expression of TRAM proteins in strain RL211 was determined using the same procedure as in strain BX04.

Assay of TRAM in Promoting Structured RNA Degradation

The PP6 RNA substrate was labeled with [γ-³²P] ATP (PerkinElmer, United States) using T4 Polynucleotide Kinase (Thermo Fisher Scientific, United States) according to the manufacturer's instruction. Radiolabeled PP6 was denatured at 85°C for 5 min and subject to a 30 min temperature-gradient descent till 10°C. 100 fmol of labeled PP6 was mixed with 1,000 pmol TRAM3066 protein in binding buffer (20 mM Tris-HCl buffer, pH 8.0, containing 5 mM MgCl₂, 10 mM KCl and 1 mM DTT). Various concentrations of RNase A (Ambion, United States) or RNase T1 (Ambion, United States) were added to the PP6-TRAM3066 mixture immediately and incubated at room temperature for 10 min. Ten microliters mixture was loaded onto a 6% or 10% polyacrylamide gel and stained with ethidium bromide (EtBr) and visualized using autoradiography.

RESULTS

Cold Induction of Archaeal TRAM Genes

Methanolobus psychrophilus R15 contains four paralogous genes encoding small TRAM proteins of approximately 7 kDa. Because transcriptomic assay showed that three (Mpsy_0643, Mpsy_3043, and Mpsy_3066) of four TRAM genes, excluding Mpsy_2002, exhibit elevated transcription (Chen et al., 2012) at lower
temperatures such as 4°C, cold shock-induced transcription of the four genes was assayed using quantitative RT-PCR (qPCR). Strain R15 was grown at 30°C in trimethylamine until the middle exponential phase and then cold shocked at 4°C for a few hours before harvesting the total RNA. Using the locus-specific PCR primers listed in Supplementary Table S2, transcript levels were quantified by qPCR. Overall, a 9- to 35-fold increase in transcription was detected for Mpsy_0643, Mpsy_3043, and Mpsy_3066 after cold shock for 1 h, whereas Student's t-test did not indicate changed transcription of Mpsy_2002 in response to temperature decrease (Figure 1A). This is consistent with the transcriptomic assay (Chen et al., 2012). Hereafter, the four TRAM proteins are denoted as TRAM0643 (Mpsy_0643), TRAM2002 (Mpsy_2002), TRAM3043 (Mpsy_3043), and TRAM3066 (Mpsy_3066).

To detect TRAM protein abundance in cold-shocked cells, we first produced two types of antibodies against TRAM2002 protein and TRAM3066-specific peptide. The antibody raised by TRAM2002 recognized all four TRAMs (Figure 1B) but only weakly to TRAM0643 and TRAM3066 (Figure 1C). Using this antibody, Western blot detected two proteins in the 30°C culture and three in the 18°C culture (Figure 1D and Supplementary Figure S1). By referenced to protein sizes, the two proteins in 30°C culture are predicted to be TRAM2002 (Mw 7.6 kDa) and TRAM3043 (Mw 7.5 kDa) indicated by arrows 1 and 2. The former was constitutively translated, while the latter was induced
confirmed that TRAM3066 was only synthesized in the 18°C culture and not in strain R15 grown at 30°C. As the TRAM2002 antibody specifically recognized its original target, and Western blot analysis showed that TRAM3066 antibody and TRAM3066 (Mw 7.2 kDa), as the two appear to be the two types of TRAMs are related to the sequences flanked by cspA, cspB, cspC, and cspE genes, and its growth below 15°C is retarded. Cultures of E. coli strains overexpressing each of Mpsy_0643, Mpsy_2002, Mpsy_3043, Mpsy_3066, and cspE were adjusted to an optical density at 600 nm of 0.9 by dilution with fresh medium. Cultures were serially diluted 10^{-5}-fold and spotted onto LB plates supplemented with ampicillin. The plates were incubated at 18, 22, and 37°C for 2–5 days with or without IPTG, as indicated (Supplementary Figure S4A). Growth at 37°C (10^{-5}), 22°C (10^{-5}), and 18°C (10^{-2}) were shown. The pNNII cultures carry the empty plasmid vector. (B) Using the antibody against TRAM2002, a Western blot was performed to determine the abundance of four TRAM proteins in E. coli BX04. The empty plasmid pNNII and bacterial CspE were included as negative controls. The amount of loaded total protein for each TRAM gene cloned strain is shown beneath the gel. ± on the top of each lane indicates the presence or absence of IPTG.

In addition to cold-induced expression, the archaeal TRAM protein shows a similar tertiary OB-fold structure to the bacterial Csps, possessing five main β-strands (Supplementary Figure S3). To determine whether the structural conservation led to functional conservation, the ability to protect the cold-sensitive E. coli strain BX04, which is unable to grow well below 15°C because of a quadruple deletion of the four csp genes of cspA, cspB, cspC, and cspE, was examined. The four TRAM-encoding genes Mpsy_0643, Mpsy_2002, Mpsy_3043, and Mpsy_3066 from M. psychrophilus R15 were each cloned into the IPTG-inducible and highly efficient expression vector pNNII. As the CspE-encoding gene from E. coli, which was included as a positive control, the four archaeal TRAM genes enabled strain BX04 to grow at low temperature, i.e., 18°C (Figure 2A and Supplementary Figure S4A), at which R15 exhibits optimal growth. In contrast, the empty pNNII vector did not allow growth. Differently, higher level of TRAM2002 and TRAM3043 appeared to poison E. coli cells; as even at 37°C, E. coli BX04 did not grow when expression of the two TRAMs was induced. However, TRAM0643 and TRAM3066 enabled BX04 growing at 18°C only when IPTG-induced to a higher concentration, TRAM0643 (12.4-fold higher protein content) and TRAM3066 (approximately 5.1-fold higher protein content) were more induced by IPTG than the other two proteins (2.1-fold). This highly increased abundance of the two TRAMs did not hurt the
E. coli, though Western blot detected relatively higher protein content for TRAM0643 than for TRAM2002 and TRAM3043 in BX04 cell (Figure 2B) by referenced to the purified four TRAMs (Figure 1C). Collectively, the four archaeal TRAMs, with slightly varied behavior between TRAM0643/3043/3066 and TRAM2002, are cold-adaptive proteins similar as the bacterial Csps.

**Archaeal TRAMs as Transcriptional Antiterminators**

The primary RNA chaperone activity of the E. coli Csp proteins is their transcriptional anttermination of nascent RNAs. To determine whether archaeal TRAMs exhibit the same activity, they were assayed using the procedure of Bae et al. (2000). In this assay, E. coli RL211, designed by Landick et al. (1990) was transformed with a plNIII vector carrying each of the IPTG-inducible four archaeal TRAM genes. Strain RL211 carries a cat gene immediately downstream of a strong trpL transcriptional terminator (Figure 3A). In the absence of an RNA chaperone to melt the terminator, cat is not expressed, and the cells are sensitive to chloramphenicol. However, if the archaeal TRAMs can melt the RNA structure in the terminator, the downstream cat gene is expressed, and the strain becomes resistant to chloramphenicol. Upon transformation with each of the four TRAM encoding genes (Mpsy_0643, Mpsy_2002, Mpsy_3043, and Mpsy_3066), the RL211 strain became chloramphenicol-resistant in the absence of IPTG (Figure 3B and Supplementary Figure S4B), whereas using IPTG induction, only Mpsy_2002 but not the remaining three TRAM gene-transformed RL211 strains showed chloramphenicol resistance. Similarly, weaker growth as strain BX04 was found for strain RL211 with expression of Mpsy_0643 and Mpsy_3043 without chloramphenicol, indicating that the two TRAMs at higher content under IPTG induction impaired E. coli RL211 as they did for strain BX04.

Using the TRAM2002 antibody, Western blot determined leaking and IPTG-induced expressions of the four TRAM genes in E. coli RL211. As shown in Figure 3C, TRAM proteins from the lpp promoter promoted leaking expression were estimated at approximately 0.1–0.3% of the total cell protein, which were calibrated based on the Western blot signal intensity of each overexpressed TRAM protein in Figure 1C. At this level, the TRAM proteins can exert a role in transcriptional anttermination in E. coli. An IPTG-induced elevation of TRAM content will poison E. coli RL211, except for TRAM2002, which appears not to be remarkably induced.
Non-sequence Specificity RNA Binding of Archaeal TRAMs

The ability of archaeal TRAMs to complement E. coli Csp proteins and lead to transcriptional antitermination suggests that they possess activity of RNA chaperone and sequence-non-specific binding to RNA. To directly test this idea, TRAM3066 was selected for a gel retardation RNA-binding assay (rEMSA), and 12 RNA Pentaprobases (PP) which are approximately 100 nt in length and encompass all possible 5 nt RNA sequences (Bendak et al., 2012), were used as binding substrates to investigate the binding specificity of the TRAM. The 3′-end-biotinylated RNA Pentaprobases were incubated with the overexpressed TRAM3066, which bound to most of the PP RNAs, except for PP4, PP7, and PP11, at appropriate ratios of 10,000 to 50,000:1 and selectively formed protein-RNA complexes in a dose-dependent manner (Figure 4A). Using the antibody generated from TRAM3066, the RNA-bound protein...
was confirmed as TRAM3066 (Supplementary Figure S5). These results suggested that TRAM3066 displays almost completely non-specific RNA recognition, which is a characteristic of RNA chaperones. Similar RNA binding was found for His6-tagged TRAM2002, but only faint RNA-protein complex bands appeared in the rEMSA (data not shown). This is most likely attributed to the contaminated RNase degradation of the RNA probe. Though a procedure to remove nucleic acids was used, RNase contamination was observed for all four TRAMs that may explain the much weaker RNA signal in the TRAM3066 complex than the free RNA probe in Figure 4A. Co-purification of RNases and TRAM suggests both proteins binding to the same RNA molecules, and adds another clue for TRAM being an RNA-associated protein.

Next, a SPR assay was employed to determine the RNA affinities of the two TRAMs more quantitatively (Figure 4B). Low affinities to RNA substrates PP1 and PP10 at $K_D$ (equilibrium dissociation constant) values ranging from 9 to 34 µM were determined for TRAM3066 and 12 to 89 µM for TRAM2002. These values were comparable but lower than those for CspA under the same condition. However, neither TRAM3066 nor TRAM2002 showed binding to ssDNA or dsDNA even up to a ratio of $10^5$ protein per RNA molecule (data not shown).

**Promotion of Structured RNA Degradation by TRAM3066**

To further validate the RNA duplex melting activity of the archaeal TRAMs, TRAM3066 was selected to test its ability to assist RNase A and RNase T1 degradation of a highly structured RNA PP6, referencing the role of *E. coli* CspA (Jiang et al., 1997). The 92-nt PP6 was 5'−radiolabelled and subjected to 85°C denaturing and 1°C gradient renaturing to 10°C to allow maximal annealing. As shown in Figure 5A, rEMSA detected a protein/RNA complex at a ratio of 5,000:1 for protein to RNA. Addition of the equivalent TRAM3066 ratio to the RNase A enzymatic mixture facilitated remarkable degradation of the structured PP6 (Figure 5B). For example, neither 6.25 pg of RNase A nor TRAM3066 alone degraded PP6 (Figure 5B, Lanes 4 and 10), but addition of TRAM3066 promoted degradation of two-thirds of PP6 by the same amount of RNase A (Figure 5B, Lane 8). Similarly, TRAM3066 facilitated RNase T1 to hydrolyse PP6 (Figure 5C). This is most likely attributed to TRAM3066 in melting the RNA secondary structure, making it more susceptible to RNase hydrolysis. This confirms the RNA chaperone activity of the archaeal TRAM.

**DISCUSSION**

This work, through studies of *in vivo* cold-induced expression and complementation of the cold-growth defect of Csp-deficient *E. coli* BX04, demonstrates that the single-domain TRAM acts as a Csp in methanogenic archaea. Three of the four TRAM genes of *M. psychrophilus* R15 are expressed either only at, or with increased expression upon reduced temperature, e.g., 18°C, and cold shock treatment induced transcription and slightly induced translation. Similar to bacterial Csps, TRAMs exhibit typical RNA chaperone activity in transcriptional antitermination, assisting RNases to degrade structured RNA and binding RNA irrespective of sequence, but with lower affinity. Given the wide distribution of TRAM across archaeal phyla (Taha et al., 2016), we speculate that this family of proteins may function as Csps in the archaeal domain. To elucidate the biological functions beyond cold protection of the archaeal TRAMs, an *in vivo* assay in a genetic tractable archaeal strain will be required.

There appear to be two types of TRAM present in cold-adaptive *M. psychrophilus* R15. TRAM2002 is constitutively expressed in response to temperature shifts and occurs as a housekeeping RNA chaperone, while TRAM0643, TRAM3043, and TRAM3066 are expressed by cold induction and function as cold stress response proteins. Considering the cellular abundance of the four TRAMs, TRAM2002 and TRAM3043 are predictably more important for the archaeon. This is similar to *E. coli*, which possesses two types of Csps: type 1 (e.g., CspA) rarely
present at 37°C and is highly induced by cold shock, while type II (e.g., CspC) occurs at both temperatures (Thieringer et al., 1998). To probe the molecular basis of the different expression modes of the two types of TRAMs, their 5' untranslated region (5'UTR) structures at 18 and 30°C were predicted. However, no temperature change-related alternative structures were found for any of the genes (Supplementary Figure S6A). This does not suggest the presence of an “RNA-thermometer” in the 5'UTRs, even though the DNA alignment showed different 5'UTR sequences for the constitutively expressed Mpsy_2002 and the other three genes (Supplementary Figure S6B). Moreover, the cellular mRNA half-life of TRAM3066 was almost the same in cold adaptive and mesophilic cultures (data not shown). Whereas, there appears to be consensus motifs present proximally to the promoters in the three cold-induced TRAMs (Supplementary Figure S2). Therefore, it is unlikely that posttranscriptional regulation, which is for the bacterial cspS, is involved in control temperature-responsive expression of methanogenic TRAMs, rather than at the transcriptional level in response to cold.

Though similar to the bacterial CspS in RNA chaperone activity, the archaeal TRAMs appear to have a lower RNA affinity than CspA determined in both rEMSA and SPR assays (Figure 4). It was determined that six residues, Trp31, Phe18, Phe31, His33, and Phe34 in CspA and CspE are key to the RNA binding activity (Supplementary Figure S3) (Newkirk et al., 1994; Feng et al., 1998), and Phe18, Phe31, and His33 are key for RNA melting (Phadtare et al., 2002). While TRAM only contains four aromatic residues analogous to the six RNA binding residues of CspS, this may be the reason of the lower RNA affinity. Protein superimposition of TRAM3066 and CspA showed that the C-terminus half fragment of TRAM is well matched to N-terminus half fragment of CspA (Supplementary Figure S3), and two of four aromatic residues of TRAM match the two CspA residues for RNA melting, thus explaining the molecular basis of TRAM in promoting structured RNA degradation (Figure 5).

In addition to cold protection, bacterial CspS display additional functions such as antioxidative stress, stationary growth and anti-antibiotic activity (Chanda et al., 2009). Given their RNA chaperone activity, the biological roles of TRAM are also predicted to extend beyond Csp functions, such as the posttranscriptional modulator CspC, which is involved in global gene regulation in E. coli (Horn et al., 2007). In our unpublished work, deletion of the only TRAM gene in Methanococcus maripaludis remarkably retarded the growth. Further study of the working mechanisms of TRAM is ongoing.

Because TRAM is a conserved domain among RNA modification proteins that are ubiquitously distributed in organisms, the findings in this work would shed light on understanding the TRAM domain in rRNA and ribosomal protein methylation in other organisms.

**AUTHOR CONTRIBUTIONS**

XD and JL designed the study. BZ, LY, LZ, LQ and JL performed experiments. XD, JL, and BZ analyzed data. XD and BZ wrote the manuscript.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.01597/full#supplementary-material

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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