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# **Roles of Cold Shock Protein 1 from *Thermus thermophilus* HB8**

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## ABBREVIATIONS

2-DE	two-dimensional electrophoresis
CD	circular dichroism
CSD	cold shock domain
Csp	cold shock protein
CT-box	the region rich in C and T bases
DTT	dithiothreitol
<i>Attcsp1</i>	deletion mutant of <i>ttcsp1</i>
<i>ecCsp</i>	<i>Escherichia coli</i> Csp
h-RNA	hairpin RNA
MALDI-TOF MS	matrix-assisted laser desorption-ionization time-of-flight mass spectrometry
PMF	peptide mass fingerprinting
RNase	ribonuclease
ssDNA	single-stranded DNA
ssRNA	single-stranded RNA
TFA	trifluoroacetic acid
<i>ttCsp1</i>	<i>T. thermophilus</i> cold shock protein 1 (TTHA0175)
<i>ttCsp2</i>	<i>T. thermophilus</i> cold shock protein 2 (TTHA0359)

## INTRODUCTION

All living cells control their cellular condition for surviving many stresses. Among the systems to respond to stress conditions, cold shock response is well studied. The first study about cold stress was taken place using *Escherichia coli* as a model organism. The protein which was over-expressed under the cold conditions in *E. coli* was named cold shock protein (Csp) (Jones, P. G. *et al.*, 1987, Goldstin, J. *et al.*, 1990). Csp comprises a family of small proteins whose amino acid sequences are highly conserved and binds to single-stranded nucleic acids via the nucleotide-binding motifs, RNP1 and RNP2 (Newkirk, K. *et al.*, 1994, Feng, W. *et al.*, 1998). In *E. coli*, nine members of Csp family proteins have been identified. Among them, *ecCspA*, *ecCspB*, *ecCspG* and *ecCspI* are highly induced by the cold stress (Lee, S. J. *et al.*, 1994, Nakashima, K. *et al.*, 1996, Wang, N. *et al.*, 1999); *ecCspD* is induced by nutrient depletion (Yananaka, K. *et al.*, 1997); and *ecCspC* and *ecCspE* are constitutively expressed at physiological temperature (Yamanaka, K. *et al.*, 1994). Some proteins belonging to CSP family, such as *ecCspC* and *ecCspE*, are considered to play not only in cold acclimation, but also in other cellular processes.

The most well-studied members of Csps are *ecCspA* and *ecCspE*. The *ecCspA* is known to have functions under the cold condition in transcriptional enhancement (Brandi, A. *et al.*, 1994), in post-transcriptional RNA defense from ribonucleases (RNases) (Ermolenko, D. N. *et al.*, 2002) and in translational enhancement. On the other hand, though the information about its functions is rather limited compared to *ecCspA*, *ecCspE* is known to unwind the RNA secondary structures and act as a transcriptional antiterminator (Phadtare, S.

*et al.*, 2002). Therefore, *ecCspE* must work under normal conditions and there are more members of Csps which work under different conditions from cold shock.

Although a significant amount of research effort has been directed towards clarifying the cellular activities of Csps as cold-dependent or cold-independent functions, no clear results have been obtained. One reason for this difficulty is the presence of several Csps in a single species e.g. *E. coli* possesses nine Csps. The manipulation of multiple genes in various combinations is still technically difficult. Moreover, *E. coli* Csps are known to work redundantly, which makes the genetic analysis problematic. In addition, Csps are apparently involved in transcription as well as post-transcriptional and/or translational processes. Because many genes might be affected by an action of Csps, genome-wide analyses need to be performed in order to elucidate cellular functions of Csps.

The structure-function relationship of Csps have also been studied. The three-dimensional structures of some Csp family proteins have been solved. Twenty-two structures have been reported from six organisms, CspA from *E. coli*, CspB from *Bacillus caldolyticus*, CspB from *Bacillus subtilis*, CspA from *Thermotoga maritima*, Csp from *Neisseria meningitidis* and CspE from *Salmonella typhimurium*. Because these proteins have the same structural features (i.e., a five-stranded  $\beta$ -barrel), it is thought that Csps are also structurally conserved. However, all of these Csps with their structures determined are cold-inducible or uncharacterized Csps. It has not been revealed whether there is a structural difference between cold-inducible and none-cold-inducible Csps.

Unlike *E. coli*, *Thermus thermophilus* HB8 has only two Csps, TTHA0175 (73 residues) and TTHA0359 (68 residues). Sequence identity between them is 69%, suggesting

functional differentiation of these Csps. If this is the case, *T. thermophilus* is potentially an extremely useful organism for studying functions of different types of Csps, that is, it may make easy to analyze the function of Csps under the normal growth condition. In addition, the proteins from *T. thermophilus* are known to be stable and suited for X-ray structural analysis. Together with the functional analysis, *T. thermophilus* Csps is also expected to contribute to study on the structure-function relationship of Csps.

Here, I studied the function and structure of *ttCsp1*, which is a non-cold-inducible protein. Specifically, I analyzed the effects of deleting *ttcsp1* on the transcriptome and proteome of *T. thermophilus*, determined the crystal structure of *ttCsp1*, compared surface charge distribution of *ttCsp1* with those of other Csps, and analyzed its affinities for oligonucleotides. Finally, I evaluate my data and discuss the new findings in terms of the likely functions of non-cold-inducible Csps.

```

ttCsp1      MQ---KGRVKWFNAEKGYGFIEREGLD-TDVFVHYTAINAK
ttCsp2      MN---KGIVKWFNAEKGYGFIQEEG-PDVFVHFSAI EAD
ecCspD      ME---KGTVKWFNNAKGFICPEGGGEDIFAHYSTIQMD
ecCspA      MSGKMTGIVKWFNADKGFGITPDDGSKDVFVHFSAIQND
ecCspB      MSNKMTGLVKWFNADKGFGISPVDGSKDVFVHFSAIQND
ecCspG      MSNKMTGLVKWFNADKGFGITPDDGSKDVFVHFTA IQSN
ecCspI      MSNKMTGLVKWFNPEKGFGITPKD GSKDVFVHFSAIQSN
ecCspC      MA-KIKGQVKWFNESKGFGITPADGSKDVFVHFSAIQGN
ecCspE      MS-KIKGNVKWFNESKGFGITPEDGSKDVFVHFSAIQTN
ecCspF      MSRKM TGIVKTFDGRKSGKGLITPSDGRIDVQLHVSALNLR
ecCspH      MSRKM TGIVKTFDRKSGKGFIIIPSDGRKEVQVHISAF TPR
          * . * * * * : : : * * * * * * : : * : : :

          GFRTLNEGDI VTFDVEPGRNGKGPQAVNVTVVEPARR 73
          GFRTLSEGERVEFEVEPEPGRNGKGPQARRVRRLL----- 68
          GYRTLKAGQSVQFDVHQGPKGNHASVIVPVEVEEAAVA 74
          GYKSLDEGQKVSFTIESGAKG--PAAGNVVTSL----- 70
          NYRTLPEGQKVTFPSIESGAKG--PAAANVVIITD----- 71
          EFRTL NENQKVEFSIEQQQRG--PAAANVVTL----- 70
          DFKTLTENQVEVEFGIENGPKG--PAAVHVVAL----- 70
          GFKTLAEGQNVFEFIQDGQKG--PAAVNVTAI----- 69
          GFKTLAEGQRVEFEITNGAKG--PSAANVIAL----- 69
          DAEIITPGLRVEFCRINGLRG--PSAANVYLS----- 70
          DAEVLI PGLRVEFCRVNGLRG--PTAANVYLS----- 70
          . : . * * * * * * * . . .

```

**Figure S1. Sequence alignment of Csps from *T. thermophilus* and *E. coli*.**

## MATERIALS AND METHODS

### 1. Materials

All strains are derivatives of *T. thermophilus* HB8 (ATCC27634). DNA-modifying enzymes, including restriction enzymes, were from Takara Bio Inc. Yeast extract and polypeptone were from Nihonseiyaku. The DNA oligomers were synthesized by BEX Co. All other reagents used were of the highest grade commercially available.

### 2. Disruption of *ttcsp1* and *ttcsp2* genes

The gene null mutants of *T. thermophilus* were constructed by using a homologous recombination method (Hashimoto, Y. *et al.*, 2004). The plasmids for gene disruption were derivatives of pGEM-T (Promega) constructed by introducing a thermostable kanamycin nucleotidyltransferase gene (*HTK*) (Hoseki, J. *et al.*, 1999) flanked by approximately 500 base pairs of DNA upstream and downstream of the *ttcsp1* and *ttcsp2* genes, respectively. For the double gene knockout, a thermostable hygromycin-B kinase gene (*hygB*) (unpublished) was used for the disruption of the *ttcsp2* gene. The wild-type strain of *T. thermophilus* HB8 was cultured in TR medium (Hashimoto, Y. *et al.*, 2004) containing 0.4 mM MgCl<sub>2</sub> and 0.4 mM CaCl<sub>2</sub> (TT medium). When the OD<sub>600</sub> value of the culture reached 0.5, 0.4 ml of the culture was incubated with 1 µg of the deletion constructs for 4 h, and transformants were isolated by positive selection on TR plates (TR medium containing 1.5 % Phytigel (Sigma-Aldrich Co.), 1.5 mM MgCl<sub>2</sub>, and 1.5 mM CaCl<sub>2</sub>) containing 50 µg/ml kanamycin or 20 µg/ml hygromycin-B. Deletion of the target gene in the chromosomal DNA was subsequently



verified by PCR analysis of genomic DNA from the mutant cells.

### **3. DNA microarray analysis**

The cells of wild type and mutants were cultured to exponential phase (OD<sub>600</sub> 0.8) in TT medium and harvested. RNA isolation to the hybridization were performed as described previously (Shinkai, A. *et al.*, 2007). For biological replication, each strain was grown three times independently, and total RNA from each sample was hybridized to distinct array. The global expression level of genes from the strains was evaluated using DNA microarray system (Affymetrix GeneChip; Affymetrix inc). The probe array was scanned with a gene array scanner (Affymetrix). The expression intensities of the 2,242 ORFs were evaluated using image data and scaled by means of the on-step Tukey bi-weight algorithm using the GeneChip Operating Software version 1.0 (Affymetrix inc). Then, the three data were normalized through the following three normalization steps using the GeneSpring GX 7.3.1 program (Agilent Tech.): data transformation (set measurements of less than 0.01 to 0.01), per chip normalization (normalize as to median), and per gen normalization (the data before cold shock was used as a control). The microarray data discussed in this study have been deposited in the NCBI gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO Series Accession No. GSE21195 and GSE21290.

### **4. Proteome analysis**

#### **4-1 Chemicals**

The chemical for mass analysis were supplied by Bruker Daltonics, Applied

Biosystem and Fluka. Trypsin Gold was purchased at Promega Co. and the calibration standard, peptide standard for MALDI-TOF were provided by Bruker Daltonics.

#### 4-2 Preparation of total cell extract of *T. thermophilus* HB8

*T. thermophilus* was cultured to prepare proteome sample for 2-DE analysis. The culture (200 mL) was harvested at 7,000 g for 10 min and the pellet was washed twice with ice-cold PBS buffer containing 1.0 mM PMSF and 2 mM EDTA to prevent proteolysis. After wash, the pellet was resuspended with 1.5 mL of lysis buffer (PBS containing 5 mM EDTA, and 1 mM PMSF, pH 8.0) and then disrupted six times by using ultrasonicator (TOMY). The crude extracts were centrifuged at 5,000 g for 10 min to remove cell debris and the supernatant was kept in -80°C for whole cell protein analysis until next experimental process. The same whole cell lysate was ultra centrifuged at 105,000 g for 1 h to separate soluble and insoluble proteins. The supernatant after ultracentrifugation was kept on -80°C as a soluble cytosolic protein sample. The pellet was washed a couple of times with cold PBS and then kept on -80°C as a insoluble membrane protein sample. The protein concentration was measured by Bradford method and 2-D Quant kit (GE Healthcare) using BSA as a standard.

#### 4-3 Two-dimensional electrophoresis (2-DE)

One miligram of protein samples were precipitated with 3 volume of ice-cold acetone to minimize contamination by such as salts in lysis buffer, phospholipids and cell constituents in the cell lysate for analytical and preparative 2-DE gel, respectively. After mixing, the samples were incubated at -30°C for 2 h and precipitated at 10,000 g for 10 min. The

precipitate was further washed two times with 5 volume of cold acetone and dried at room temperature completely. After drying the sample, the protein pellet was suspended with 250 uL (for analytical gel) and 500 uL (for preparative gel) of activated rehydration buffer (8 M urea, 30% glycerol, 2% m/v CHAPS, 10 mM DTT, 0.5% IPG buffer and trace of bromophenol blue). Subsequently the sample was completely dissolved by vigorous vortexing and undissolved protein pellets were precipitated and eliminated by centrifugation at 14,000 g for 10 min at room temperature.

Total 250 uL and 500 uL of protein samples dissolved in rehydration buffer were applied into the 24 cm versatile strip holder (Amersham-Pharmacia) for analytical and preparative samples, respectively. Thirteen and twenty-four centimeter Immobiline DryStrip (linear pH 4-7 or non-linear pH 3-10, Amersham-Pharmacia) were employed for the first electro-focusing process with a IPGPhor II apparatus (Amersham-Pharmacia). Proteins were adsorbed onto the dry strip and strip gels were also rehydrated for 12 h at 20°C. For analytical 2-DE gel, the voltage set was 0.2 kV (6 h), 0.5 kV (1 h), 1.0 kV (1 h), until 8.0 kV (gradient 1 h) and 8.0 kV (upto 20 kVh); for preparative 2-DE gel, the voltage set was 0.2 kV (2 h), 0.5 kV (6 h), 1.0 kV (2 h), 3.0 kV (2 h), until 8.0 kV (gradient 1h) and 8.0 kV (upto 80 kVh). Prior to the second dimension SDS-PAGE, focused IPG strips were equilibrated for 15 min twice with 1% DTT and 2.5% iodoacetamide containing equilibration solution (7 M urea, 2% SDS, 30% glycerol and 50 mM Tris-HCl, pH 6.8), respectively. The second dimension PAGE was carried out on a 12.5% polyacrylamide linear gradient gels (13 cm x 15 cm x 1 mm for analytical gel and 25.5 cm x 20.5 cm x 1 mm for preparative gel) with constant voltage of 100 V and 2.5W/gel for 30 min then 100 W until the dye eluted from the

bottom of the gel. Protein spots on the both analytical and preparative gels were detected by a silver staining method (GE Healthcare) or highly sensitive blue silver staining method using colloidal coomassie brilliant blue G-250 (Candiano, G. *et al.*, 2004). 2-DE maps were obtained in triplicate for each analyzed sample for biological implication. Protein spots detected in all gels were analyzed for comparative expression study.

#### 4-4 Comparative analysis of 2-DE map

The ImageMaster 2-D Platinum software package (GE Healthcare) and ImageScanner (GE Healthcare) were employed for the statistical data analysis with spot detection, pair matching and comparing analysis of up- and down-regulated proteins. 2-DE images were acquired with 300 dpi resolution as Melanie format and then background of each image was subtracted. Quantitative difference between each proteome map of *T. thermophilus* wild type and  $\Delta ttcsp1$  mutant stains was evaluated by three independent analytical 2-DE PAGE gels. The significant differences ( $p < 0.05$ ) of expression of each protein spots between data were tested by Student's *t*-test. The increasing and decreasing index (fold change) was calculated as ratio of averaged spot intensities (relative % volume) between the investigated and control 2-DE maps.

#### 4-5 Protein in-gel digestion and MS analysis

The protein spots from 2-DE PAGE gel were excised and washed twice with 50 mM ammonium bicarbonate buffer, pH 8.0 and 100% acetonitrile alternatively. After wash with acetonitrile, shrank gel particle was completely dried in the speed-vacuum drier and then add

1.5  $\mu$ L of 15 ng trypsin solution. After the gel particle was fully rehydrated under ice for more than 30 min, 6.5  $\mu$ L of trypsin digestion buffer (40 mM ammonium carbonate, pH 8.0, and 10% acetonitrile) was added and incubated for overnight on the 37°C. One microliter of peptide mixtures extracted from 2-DE spot were mixed with equal volume of matrix solution that was prepared freshly by dissolving 0.2 mg  $\alpha$ -cyano-4-hydroxycinnamic acid (CHCA) in 1 ml of 80% acetonitrile solution containing 0.1% trifluoroacetic acid (TFA) and then, 0.5  $\mu$ L of mixed solution were crystallized onto a 600 nm Anchor Chip (Bruker Daltonics). After dried and crystallized peptide/matrix mixtures on AnchorChip at the room temperature, it was washed with 0.1% TFA and re-dried at room temperature for analysis. The samples were analyzed with an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics) under reflector mode to collect peptide mass spectra for peptide mass fingerprinting (PMF) analysis. The mass spectra of tryptic digested peptides were acquired as the average of the ion signals which generated by the irradiation of the target with 100–150 times of laser pulses, in positive reflect mode with 20-25 kV voltage. For mass calibration (error tolerance < 10 ppm), peptide standard mixture mono (Bruker Daltonics) was engaged for external calibration. For MS/MS analysis, lift mode was employed with 25-30 kV voltage and post source decay fragments ion spectra were acquired after isolation of the appropriate parental ions. The mass spectra data produced in both reflect and lift mode were elaborated by using the FlexControl 2.2 (Bruker Daltonics). FlexAnalysis 2.2 (Bruker Daltonics) and Biotool 2.2 software (Bruker Daltonics) were used to process and collect PMF spectra obtained from each tryptic digested protein. Mascot (Matrix science) in-house version software was employed to identify the spots from *T. thermophilus* genome database in a local Mascot server by PMF

analysis. Database search parameter for PMF analysis allowed to methionine oxidation, cysteine carbamidomethylation and maximum missing cleavage, one for variable modification with 0.2 Da peptide error tolerance. A theoretical *pI* and *Mw*. were calculated by Biotoools 2.2 program and functional classification was followed by KEGG classification (<http://www.genome.jp/kegg/pathway.html>).

## 5. X-ray crystallography

### 5-1 Protein overexpression and purification

Sequence data of the *ttha0175* gene coding for *ttCsp1* was obtained from the *T. thermophilus* HB8 genome project (DDBJ/EMBL/GeneBank AP008226). The DNA fragment containing *ttcsp1* gene was amplified by PCR using the genomic DNA as a template with *Taq* DNA polymerase, forward (5'-ATATCATATGCAAAAGGGTCGGGTCAAGTGGTTCA-3'), and reverse (5'-ATATGGATCCTTATTAGCGCCGCGGGCTCCACCAC-3') primers. To improve the gene expression, the second codon CAG was changed to CAA according to the effect of second codon variants on the expression of the *lacZ* gene in *E. coli* (Looman, A. C. *et al.*, 1987). The amplified fragment was digested with the restriction enzymes *NdeI* and *BamHI*, and the ORF was ligated into the compatible sites of the expression vector pET-11a. Sequence analysis revealed that the construction was error free. *E. coli* Rosetta(DE3) cells transformed with the resultant pET-11a/*ttcsp1* plasmid were cultured at 37°C for 20 h in LB media containing 50 µg/ml ampicillin and were harvested by centrifugation.

Frozen cells (8.4 g) were thawed, suspended in 70 ml of buffer I (20 mM Tris-HCl and

50 mM NaCl, pH 8.0) and sonicated on ice for 10 min with an ultrasonic disrupter. Then, 70 ml of pre-warmed buffer I was added to the suspension. The cell extract was incubated at 70°C for 10 min and then centrifuged at 40,000 g for 60 min at 4°C. After this step, most of *E. coli* proteins were excluded as precipitates. The supernatant was loaded onto a TOYOPEARL SuperQ-650M column (Tosoh) equilibrated with buffer II (20 mM Tris-HCl, pH 8.0). The column was washed with 100 ml of buffer II and the fractions passed through the column were collected. The fractions containing *ttCsp1* were desalted and loaded onto a Reource S column (GE Healthcare) equilibrated with buffer III (20 mM MES, pH 6.0). The column was washed with buffer III and the proteins were eluted with a linear gradient of 0–0.4 M NaCl in 120 ml of buffer III. The fractions containing *ttCsp1* were concentrated and loaded onto HiLoadd 16/60 Superdex 75pg (GE Healthcare) equilibrated with 20 mM Tris-HCl and 150 mM NaCl, pH 8.0. The fraction containing *ttCsp1* were concentrated and stored at –4°C. The purity of the protein was assessed by SDS-PAGE in each step.

## 5-2 Crystallization and data collection

An initial attempt of *ttCsp1* crystallization was performed with the sitting-drop vapor-diffusion method at 293 K and 480 conditions using 8 screening kits (Hampton Research and Emerald Biosystems). Drops were prepared by mixing 0.5  $\mu$ l protein (17.0 mg/ml) solution with 0.5  $\mu$ l reservoir solution and equilibrated against 200  $\mu$ l of reservoir solution. Initial crystallization trials produced several crystal forms using solutions containing polyethylene glycol. The reservoir solution that gave best crystallization result contained 0.1 M Tris-HCl (pH 8.2) and 32% PEG1500.

X-ray diffraction data were collected at the RIKEN Structural Biology Beamline II (BL44B2) at SPring-8 (Hyogo, Japan) (Adachi, S *et al.*, 2001). The crystal was mounted on the goniometer at 100 K by and the crystal-to-detector distance was 150 mm. A total of 360° of data was collected with an oscillation angle of 1° and an exposure time of 10 sec per degree of oscillation. The collected data were processed with the HKL2000 suite (Otwinowski, Z. *et al.*, 1991) and the data collection statistics were summarized in Table 6-1.

### 5-3 Structure determination and analysis

The structure of *ttCsp1* was determined by the molecular-replacement method using *MOLREP* (Vagin, A. *et al.*, 1997) from the CCP4 package (Collaborative Computational Project, Number 4, 1994). Among cold shock proteins whose structures are available, *Bacillus caldolyticus* cold shock protein (Bc-Csp) mutant V64T/L66E/67A showed the highest sequence homology to *ttCsp1*. Hence, the coordinate of Bc-Csp mutant (PDB code, 1HZA; Delbrück, H. *et al.*, 2001) was used as the search model. A solution was found with two molecules in the asymmetric unit with an R factor of 0.492. CNS (Brüger, A. T. *et al.*, 1998) was used to refine the atomic positions and thermal factors. 10% of the data was used for calculation of  $R_{\text{free}}$  during the refinement with CNS. After initial rigid body and simulated annealing refinement, the automatic-tracing procedure in ARP/wARP (Parrakis, A. *et al.*, 2001) was used to build a main-chain model for 124 of the 146 amino acid residues. The rest of the molecule was build into the electron density map using XtalView/X-fit (McRee, D. E., 2000) and model refinement was performed with CNS. The final model was validated using the program *PROCHECK* (Laskowski, R. A, *et al.*, 1997) in the CCP4



package. The refinement statistics are summarized in Table 5-1.

Least squares comparison of two structures and calculation of the root-mean-square (r.m.s) deviations of the main chain atoms were carried out using LAQKAB in CCP4 (Kabsh, W. 1976). The average structures were used in the case of the structures which were determined by NMR and calculated using CNS. Figures were drawn using the programs PyMOL (DeLano, W. L., 2002).

A model structure of *ttCsp1* complexed with dT6 was constructed on the basis of bcCsp (2HAX) and dT6 (Max, K. E., 2007). Because two bcCsp molecules (A and B chains) form a domain-swapping dimer, the main-chain atoms were superimposed using LSQKAB as follows: residues 2–21 of *ttCsp1*, 2–21 of bcCsp A chain; 25–37 of *ttCsp1*, 25–37 of bcCsp A chain; 38–55 of *ttCsp1*, 38–55 of bcCsp B chain; and 58–68 of *ttCsp1*, 56–66 of bcCsp B chain.

#### 5-4 Comparison of surface charges

The structural models of 15 Csps were obtained using the SWISS-MODEL (SIB) and ClustalW2 programs (EBI) using *ecCspA* as a template. The surface charges of the molecules were calculated using the APBS1.3 program.

## 6. Oligonucleotide-binding analysis

### 6-1 Nucleic Acid Binding Measured by Fluorescence Quenching

Fluorescence spectra were recorded with a Hitachi fluorescence spectrophotometer, model F4500. The protein solution contained 0.5–0.48  $\mu$ M Csp1, 25 mM Tris-HCl, 100 mM

KCl, and 0.5 mM EDTA, at pH 7.5. This protein solution was successively titrated with ssDNA or ssRNA solution in a 5 x 5 mm cell at 25°C. The excitation and emission wavelengths were 295 and 350 nm, respectively. The nucleotide sequences of ssDNA and ssRNA used in this experiment are shown in Table 6-1. LoopdT7, loopdT7dA4, loopdT11, stem5dT7, stem3dT7, LoopU7, stem5U7 and stem3U7 were prepared by incubating at 95°C (ssDNA) or 80°C (ssRNA) for 2 min and 60°C for 10 min to form secondary structure as shown in Figure 6-1.

The  $K_d$  values for high-affinity oligonucleotides were determined by competition experiments with 10  $\mu$ M dT4. The equilibrium dissociation constant,  $K_d$ , was determined as follows. The fluorescence quenching was analyzed assuming the following scheme for the interaction of *t*Csp1 (P) with ssDNA or ssRNA ligand ( $S_2$ ) (Fersht, A. R., 1999),



*t*Csp1 (P) binds to a competitor dT4 ( $S_1$ ) to form the complex  $PS_1$  with dissociation constant  $K_{PS1} = [P][S_1] / [PS_1]$ . *t*Csp1 competitively binds to a ligand ( $S_2$ ) to form the complex  $PS_2$  with dissociation constant  $K_{PS2} = [P][S_2] / [PS_2]$ . Because the fluorescence intensity of oligonucleotide is nearly zero, the observed fluorescence intensity ( $F_{obs}$ ) is expressed as follows:

$$F_{obs} = F_P[P] + F_{PS1}[PS_1] + F_{PS2}[PS_2] \quad (3)$$

where  $F_P$ ,  $F_{PS1}$ , and  $F_{PS2}$  are the molar fluorescence intensities of P,  $PS_1$ , and  $PS_2$ , respectively.

The total concentrations of P,  $S_1$ , and  $S_2$  are expressed by (4), (5), and (6), respectively.

$$[P]_t = [P] + [PS_1] + [PS_2] \quad (4)$$

$$[S_1]_t = [S_1] + [PS_1] \quad (5)$$

$$[S_2]_t = [S_2] + [PS_2] \quad (6)$$

From  $K_{PS1}$  and (5),

$$[S_1]_t = [S_1] + [PS_1] = \frac{K_{PS1}[PS_1]}{[P]} + [PS_1] = \left(\frac{K_{PS1}}{[P]} + 1\right)[PS_1] \quad (7)$$

Then,

$$[PS_1] = \frac{[P]}{[P] + K_{PS1}} [S_1]_t \quad (8)$$

From  $K_{PS2}$  and (6),

$$[PS_2] = \frac{[P]}{[P] + K_{PS2}} [S_2]_t \quad (9)$$

From (4), (8) and (9),

$$\begin{aligned} [P]_t &= [P] + [PS_1] + [PS_2] \\ &= [P] + [S_1]_t \left(\frac{[P]}{[P] + K_{PS1}}\right) + [S_2]_t \left(\frac{[P]}{[P] + K_{PS2}}\right) \end{aligned} \quad (10)$$

Therefore,

$$\begin{aligned} & -([P] + K_{PS1})([P] + K_{PS2})[P]_t + ([P] + K_{PS1})([P] + K_{PS2})[P] \\ & + [S_1]_t[P]([P] + K_{PS2}) + [S_2]_t[P]([P] + K_{PS1}) = 0 \end{aligned} \quad (11)$$

By using this  $[P]$  value obtained from (11),  $[PS_1]$  and  $[PS_2]$  were obtained from (8) and (9), respectively. For the competition experiments, (3) was fitted to the observed data by fixing  $F_P$ ,  $F_{PS1}$ , and  $K_{PS1}$ , using Igor Pro software (WaveMetrics). The constructed theoretical curves are shown in Figure 6-3.

## 6-2 Analytical Size-exclusion Chromatography

- (1) The oligomeric structure of *t*Csp1 in the presence or absence of nucleic acid

ligand and (2) the number of nucleotide molecule bound to *ttCsp1* were analyzed by size-exclusion chromatography.

The Superdex 75 10/300 GL column (GE healthcare) was pre-equilibrated with 20 mM Tris-HCl and 200 mM NaCl at pH 8.0. In the presence of oligonucleotide, the reaction mixture contained Csp1 (47  $\mu$ M) and dT7 (47  $\mu$ M) or dT31 (8  $\mu$ M) in the same buffer, and was incubated for 5 min before applying to the column. The eluent from the column was monitored by the UV-absorption at 280 and 260 nm. The apparent molecular mass was estimated by comparing its retention time with those of molecular mass markers (Sigma).

## 7. RNase assay

A 50-mer hairpin RNA (h-RNA, 5'-CCCCCGGGGGGGGGGAUUCG UUA UUCAACCUCCCCCCCCCCCCCCCC-3') was chemically synthesized and was radiolabelled at the 3'-end with [ $\gamma$ -<sup>32</sup>P]ATP using T4 RNA ligase. h-RNA forms a hairpin structure shown in Figure 7-1. *ttCsp1* was incubated with 0.1  $\mu$ M h-RNA at 37°C for 20 min. After incubation, 0.3  $\mu$ M TTHA0252, a single-strand-specific 5' to 3' exonuclease, was added. Aliquots of the reaction mixture was removed at various times to stop the reaction by adding the denaturing dye (5 mM EDTA, 80% deionized formamide, 10 mM NaOH, 0.1% bromophenol blue and 0.1% xylene cyanol) and heat-treatment at 95°C for 5 min. The reaction mixtures were loaded onto 25% acrylamide gels (8M urea and x 1 TBE buffer (89 mM Tris-borate, 2 mM EDTA)) and electrophoresed with x 1 TBE buffer. The gel was dried and placed in contact with an imaging plate. The bands were visualized and analyzed using a BAS2500 image analyzer (Fuji film).

## RESULTS AND DISCUSSION

### 1. Cell viability of *csp* deletion mutants

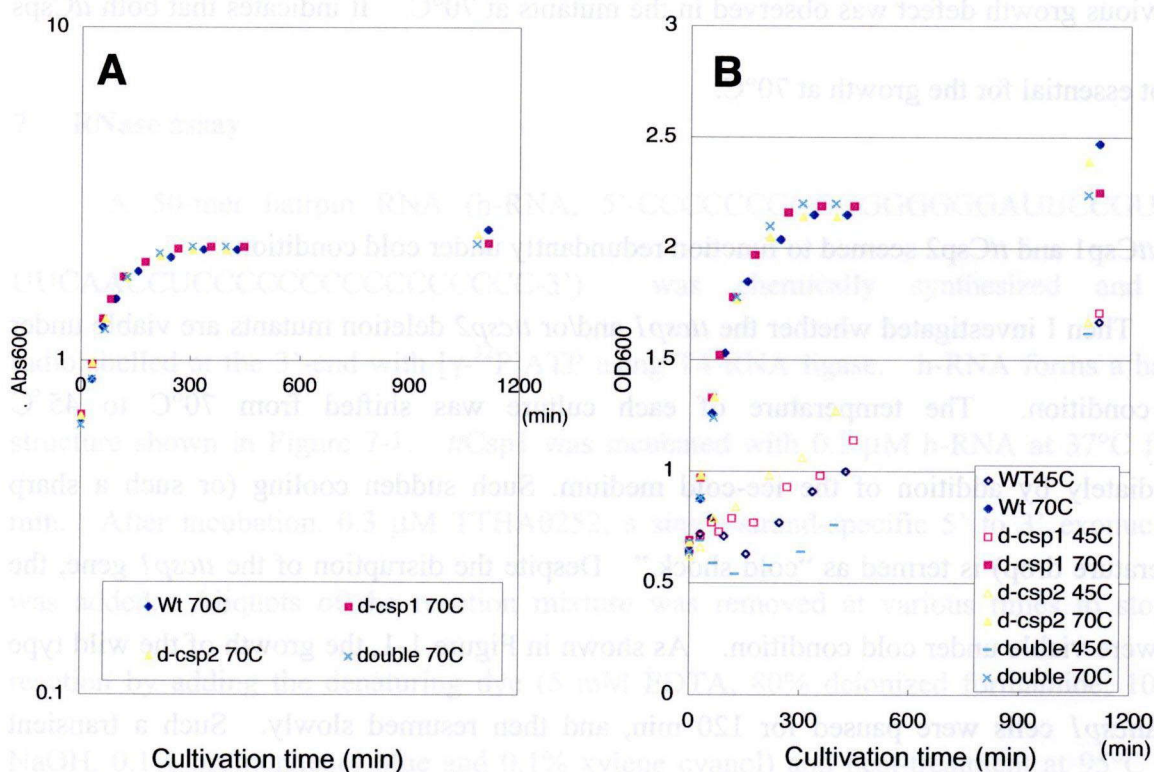
#### 1.1 Both *ttCsp1* and *ttCsp2* were not essential for the growth at 70°C.

When plasmids for producing the *ttcsp1* and/or *ttcsp2* deletion mutants described in Materials and Methods transformed the wild type cells, the transformants formed colonies on the TT plates (rich media) containing selection markers at 70°C. As shown in Figure 1-A, no obvious growth defect was observed in the mutants at 70°C. It indicates that both *ttCsp*s are not essential for the growth at 70°C.

#### 1.2 *ttCsp1* and *ttCsp2* seemed to function redundantly under cold condition.

Then I investigated whether the *ttcsp1* and/or *ttcsp2* deletion mutants are viable under cold condition. The temperature of each culture was shifted from 70°C to 45 °C immediately by addition of the ice-cold medium. Such sudden cooling (or such a sharp temperature drop) is termed as “cold shock.” Despite the disruption of the *ttcsp1* gene, the cells were viable under cold condition. As shown in Figure 1-1, the growth of the wild type and  $\Delta$ *ttcsp1* cells were paused for 120 min, and then resumed slowly. Such a transient growth arrest has been known as a typical cold shock response in many bacteria (Horn, G. *et al.*, 2007). The  $\Delta$ *ttcsp1* mutant could adapt to the cold condition and re-grow in the same manner as the wild type. No growth defect was observed in the  $\Delta$ *ttcsp1* mutant under the cold condition. On the other hand, the growth of the  $\Delta$ *ttcsp2* cells exhibited very shorter

growth arrest compared to the wild type and  $\Delta ttcsp1$  cells. The double deletion mutant could not grow at 45°C after the temperature downshift and a substantial portion of the cells appeared to die. Cells grown after the cold shock may be revertants. The observation that the  $ttcsp1/ttcsp2$  double deletion strain was lethal indicates that at least one Csp homologue is essential for the survival of *T. thermophilus* HB8 at low temperatures. Since the  $\Delta ttcsp2$  cells did not exhibit a typical cold shock response,  $ttcsp2$  was considered to be more important in cold adaptation of the organism.



**Figure 1-1. Growth curves of the wild type and the  $ttcsp$  deletion mutant strains.**

The values of the absorbance at 600 nm were measured at the time points indicated.

(A) WT (◆), the  $\Delta ttcsp1$  mutant (■), the  $\Delta ttcsp2$  mutant (▲) and the  $\Delta ttcsp1\Delta ttcsp2$  double mutant (×) were grown at 70°C

(B) WT (◆/◇), the  $\Delta ttcsp1$  mutant (■/□), the  $\Delta ttcsp2$  mutant (▲/△) and the  $\Delta ttcsp1\Delta ttcsp2$  double mutant (×/—) were grown at (70°C /45°C).

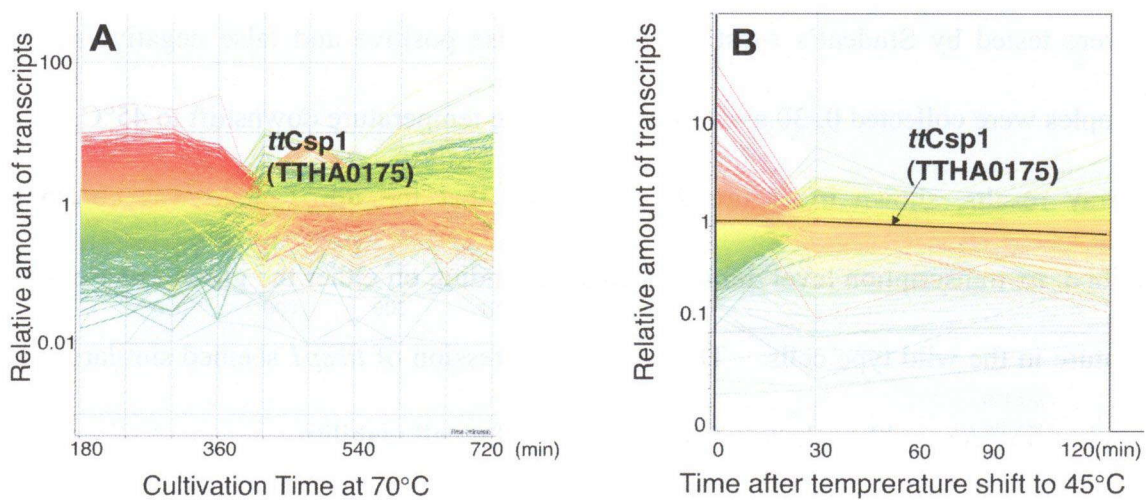
## 2. DNA microarray analysis

### 2.1 *ttCsp1* was constitutively expressed even under the cold condition.

Since the viability of the *ttcsp1* deletion mutant provided no functional information, I examined the change in the transcriptional level of *ttCsp1* in the wild type cells upon temperature downshift. DNA microarray analysis was performed using Affymetrix GeneChip system and GeneSpring software. The array signals were normalized to the value of 50 percentile per Chip. I selected the genes whose normalized signals were changed more than 2-fold. I prepared three independent samples sets, and the significant changes ( $p < 0.05$ ) were tested by Student's *t*-test to eliminate false positive and false negative results. The samples were collected 0, 30 and 120 min after the temperature downshift to 45°C. The microarray results, shown in Figure 2-1, indicated that the *ttcsp1* gene was constantly transcribed: its transcription level did not change depending on either the growth phase or the temperature in the wild type cells. The constant expression of *ttcsp1* seemed similar to that of housekeeping gene, although *ttcsp1* is not essential for cell viability.

It should be noted here that cell cultures were centrifuged for 5 min at 4°C when I harvested the cells for the microarray analysis. This means that the cells were subjected to temporary cold shock during the centrifugation. To check the effects of this short cold shock to the microarray data, I prepared two samples of cell culturers: one was prepared as described above, whereas the other was fixed by adding cold ethanol (final 50%) before the centrifugation. The results of these two samples shown in Figure 2-2 indicated that the temporary cold shock treatment did not affect the transcriptional transient levels of all genes

except two genes, *ttha0359* and *ttha0948*. *ttha0359* and *ttha0948* code for *ttCsp2* and fatty acid desaturase, respectively. Therefore, it was validated that the transcription of *ttcsp1* was not affected by temporal cold shock upon harvesting the cells. It should also be mentioned here that the transcription of mRNA for *ttCsp2* and fatty acid desaturase initially responded to the cold stress.



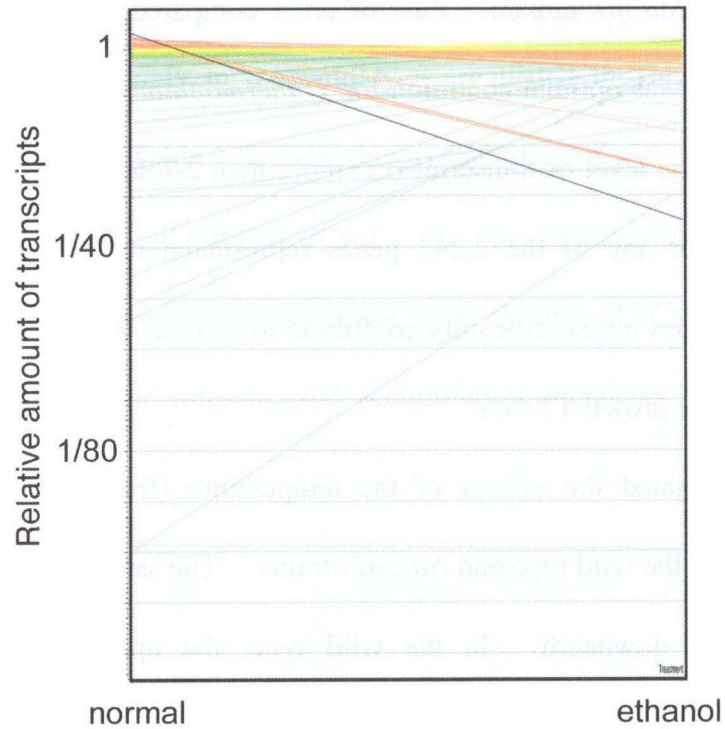
**Figure 2-1. Expression profile of *ttcsp1* gene.**

(A) Expression profile of the wild type during the growth at 70°C.

(B) Expression profile of the wild type after temperature downshift to 45°C.

The profile for the *ttcsp1* gene is indicated as black lines. Green, yellow, and red lines represent increased expression, no change, and decreased expression during culture, respectively.





**Figure 2-2. Effect of the temporary cold shock treatment on the microarray signals.**

The array signals from the samples prepared by different ways was compared. The signals of those prepared by normal way, centrifugation at 4°C for 5 min, are shown on the left axis, whereas those prepared by fixation with ethanol before centrifugation are shown on the right axis. The signals of *ttCsp2* and fatty acid desaturase are represented as thick black line and thick red line, respectively.

### 2.3 *t*Csp1 might control the transcription of some genes required for survival.

To obtain some clues to the *t*Csp1 function, I investigated the transcriptional profiles of the wild type and  $\Delta ttcsp1$  mutant. First of all, I compared the profiles from the cells grown at 70°C, which was optimal condition for *T. thermophilus* HB8. To my surprise, no significant change in the level of transcript (i.e. more than 2-fold in the  $\Delta ttcsp1$  mutant,  $q < 0.05$ ) was detected for any of the 2,242 genes represented on the array. This finding suggests that *t*Csp1 does not significantly contribute to cellular transcriptional control at the optimal temperature for growth (70°C).

Next I investigated the effects of the temperature (from 70°C to 45°C) on the transcriptional level in the wild type and  $\Delta ttcsp1$  strains. The samples were prepared 30 min after the temperature downshift. In the wild type, the up-regulated (75 genes) and down-regulated (76 genes) genes were identified as listed in Tables 2-1 and 2-2. Similar transcriptional changes are reported for other bacteria under the cold stress condition. These results suggest that cold adaptation mechanisms of *T. thermophilus* HB8 is not so different from those of other bacteria.

Then I analyzed the microarray data of the  $\Delta ttcsp1$  mutant under the same conditions (from 70°C to 45°C). Tables 2-5 and 2-6 are the lists of up- and down-regulated genes at 45°C in the  $\Delta ttcsp1$  mutant. The numbers of the up- and down-regulated genes are 136 and 139, respectively, which were larger than those in the wild-type. The decrease in the expression level in the  $\Delta ttcsp1$  mutant implies that *t*Csp1 is involved in alteration of transcription upon the cold shock. *t*Csp1 might support initiation or continuation of transcription of the genes listed in Table 2-6. Inversely, *t*Csp1 might inhibit the

transcription initiation and elongation, or support the mRNA degradation of the genes listed in Table 2-5.

Next I compared the results of the wild type with those of  $\Delta ttcsp1$ . Cold-inducible and cold-repressible genes only in the wild type are listed in Table 2-3 and Table 2-4, respectively. As shown in Table 2-3, some factors including *ttCsp2* needed to adjust to cold condition could not be induced without *ttCsp1*. On the contrary, many factors function in metabolism could not be repressed by cold shock in the absence of *ttCsp1* (see Table 2-4). It implies that *ttCsp1* has some roles in cold acclimation and delay of cell division in the transcriptional level.

**Table 2-1. The genes increased under the cold condition in the wild type.**

Function	Gene Name	fold	Description
Genetic Information Processing	TTHA1002	3.9	response regulator
	TTHA1003	3	sensor histidine kinase
	TTHA1502	2.8	response regulator
	TTHA1036	2.7	signal recognition particle protein
	TTHB016	2.7	oxidoreductase, short-chain dehydrogenase/reductase family
	TTHA0008	2.4	phage shock protein A
Chaperones	TTHB117	3.4	putative type IV pilin
	TTHB119	2.6	prepilin-like protein
Csp2	TTHA0359	2.7	cold shock protein
detox	TTHB118	3.1	probable secretion system protein
DNA repair/ recombination/ replication	TTHA1440	2.4	excinuclease ABC subunit A (UvrA)
	TTHB201	2.4	transposase-like protein
	TTHA0112	2.2	endonuclease III
	TTHA1539	2.2	putative phage integrase/recombinase
RNA helicase transcription	TTHA0109	2.4	ATP-dependent RNA helicase
	TTHA0622	3.5	transcription elongation factor GreA
translation/ ribosomal protein	TTHB023	4.7	transcriptional regulator, TetR family
	TTHA1138	2.1	30S ribosomal protein S15
Metabolism of nucleotide	TTHA1442	4.1	glucose inhibited division protein
	TTHA0135	2.8	MutT/nudix family protein
	TTHA1920	2.3	thioredoxin reductase
Membrane/ Transporter/ Lipoprotein	TTHA1004	4.5	conserved hypothetical membrane protein
	TTHA1008	4.4	ABC transporter, permease protein
	TTHA1007	3.6	ABC transporter, ATP-binding protein
	TTHA0377	3.3	sugar ABC transporter, permease protein
	TTHA1391	2.9	GTP-binding protein
	TTHA0725	2.6	membrane-bound protein LytR
	TTHA0194	2.4	probable tripartite transporter, small subunit
Metabolism of ammino acid	TTHA1914	2.6	homocitrate synthase
	TTHA1743	2.5	orotidine 5'-phosphate decarboxylase
	TTHA1320	2.4	periplasmic serine protease
	TTHA0957	2.2	dihydrodipicolinate synthase
	TTHA0958	2.1	2-hydroxyhepta-2,4-diene-1,7-dioate isomerase/ 5-carboxymethyl-2-oxo-hex-3-ene-1,7-dioate decarboxylase
	TTHA0760	2	O-acetyl-L-homoserine sulfhydrylase
Metabolism of Carbohydrate	TTHA1066	3.6	probable transaldolase
	TTHB019	2.5	MaoC-related acyl dehydratase
	TTHA0022	2.4	glucose-1-phosphate adenyltransferase
	TTHB017	2.3	medium-chain acyl-CoA ligase-related protein
	TTHA1261	2.2	4-alpha-glucanotransferase (amylomaltase) (disproportionating enzyme) (D-enzyme)
Metabolism of energy	TTHA0021	2.1	putative NADPH oxidoreductase
Metabolism of fatty acid	TTHB022	5.1	putative acyl-CoA dehydrogenase
	TTHA0750	4	3-oxoacyl-[acyl carrier protein] reductase
	TTHB015	3.4	putative acyl-CoA dehydrogenase
	TTHB020	3.4	3-oxoacyl-[acyl carrier protein] reductase
	TTHA0948	2.2	fatty acid desaturase

Others /Unknown	TTHA0838	2.5	glutaredoxin-like protein
	TTHA0840	2.5	probable thiol:disulfide interchange protein
	TTHA0929	2.1	phosphopantetheine adenylyltransferase
	TTHA1162	2.4	excisionase domain protein
	TTHA1217	2.2	prepilin-like protein
	TTHA1369	2	phospholipase domain protein
	TTHA1511	2.9	putative catechol 1,2-dioxygenase
	TTHA1512	2.8	putative nucleotidyltransferase
	TTHB012	4.1	phosphoglycerate mutase family protein
	TTHB014	3.7	probable phosphotransferase
	hypothetical protein	TTHA0025	2.3
TTHA0105		2.2	hypothetical protein
TTHA0163		2.9	hypothetical protein
TTHA0592		2.4	hypothetical protein
TTHA0613		2	hypothetical protein
TTHA0777		2.1	hypothetical protein
TTHA0808		2.1	hypothetical protein
TTHA0828		2.6	hypothetical protein
TTHA1001		8	hypothetical protein
TTHA1005		4.1	hypothetical protein
TTHA1006		3.9	hypothetical protein
TTHA1469		2	hypothetical protein
TTHA1510		2.1	hypothetical protein
TTHA1540		2.1	hypothetical protein
TTHA1581		2.7	hypothetical protein
TTHA1734		2	hypothetical protein
TTHB003		3	hypothetical protein
TTHB013		4.2	hypothetical protein
TTHB021		3.6	hypothetical protein
TTHB120		2.1	hypothetical protein

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**Table 2-2. The genes decreased under the cold condition in the wild type.**

Function	Gene Name	fold	Description
Genetic Information Processing	TTHA0829	3.1	putative acetoin utilization protein, acetoin dehydrogenase
	TTHB186	2.9	putative transcriptional regulator
	TTHB073	2.9	transcriptional regulator
	TTHA0058	2.2	transcriptional repressor of class I heat-shock genes
	TTHA1498	3.4	elongation factor G (EF-G-2)
LytR/CspA/Psr family	TTHA1778	2.4	LytR/CspA/Psr family protein
Metabolism of nucleotide	TTHA0735	2.2	cytidine deaminase
Membrane/ Transporter/ Lipoprotein	TTHA1141	6	cation-transporting ATPase
	TTHA1408	4.8	ABC transporter, ATP-binding protein
	TTHA1407	3.9	cytochrome c-type biogenesis protein, heme exporter protein B
	TTHB076	3.4	putative C4-dicarboxylate transporter, large permease protein
	TTHA1137	3	major facilitator superfamily transporter
	TTHA0452	2.5	branched-chain amino acid ABC transporter, ATP-binding protein
	TTHB218	2.2	ABC transporter, ATP-binding protein
	TTHA0451	2.2	probable branched-chain amino acid ABC transporter, amino acid binding protein
	TTHA1337	2.1	peptide ABC transporter, permease protein
	TTHA1338	2	ABC transporter permease protein
Metabolism of amino acid	TTHA0606	2	aspartate 1-decarboxylase
Metabolism of Carbohydrate	TTHA1836	15	isocitrate lyase
	TTHA0506	3.1	malate synthase
	TTHA0097	2.3	NADH-quinone oxidoreductase chain 14
Metabolism of Cofactors and Vitamins	TTHA1506	3.8	glutamyl-tRNA reductase
	TTHA0190	2.1	dihydroneopterin aldolase
	TTHA1775	2	pantoate--beta-alanine ligase
Metabolism of energy	TTHA1133	20	ba3-type cytochrome c oxidase polypeptide IIA
	TTHA1134	14	ba3-type cytochrome c oxidase polypeptide II
	TTHA1942	13	putative cytochrome c oxidase assembly protein
	TTHA1447	12	alanine dehydrogenase
	TTHA1135	9.6	ba3-type cytochrome c oxidase polypeptide I
	TTHA0663	3.4	pyruvate orthophosphate dikinase
	TTHA1117	2.9	iron-sulfur protein
	TTHA1505	2.7	cytochrome c assembly protein-related protein
	TTHA0310	2.6	cytochrome c oxidase assembly factor (CtaA) protoheme IX farnesyltransferase (CtaB)
	TTHA1276	2.2	V-type ATP synthase subunit E
	TTHA1272	2.1	V-type ATP synthase subunit B
	TTHA1271	2	V-type ATP synthase subunit D
	TTHA1273	2	V-type ATP synthase subunit A
Metabolism of fatty acid	TTHB116	2.2	acyl-CoA dehydrogenase, short-chain specific (probable AidB protein)
	TTHA0604	2	medium-chain-fatty-acid--CoA ligase
Others /Unknown	TTHA0982	2.7	bacterioferritin
	TTHA0642	2.4	putative glycosyltransferase
	TTHA1448	4.2	4-hydroxybutyrate CoA-transferase
	TTHB010	2.6	transposase-related protein
	TTHA1335	2.2	branched-chain amino acid ABC transporter, ATP-binding protein

hypothetical protein

TTHA0236	2.3	hypothetical protein
TTHA0268	2.1	hypothetical protein
TTHA0316	16	hypothetical protein
TTHA0467	3.2	hypothetical protein
TTHA0487	2	hypothetical protein
TTHA0646	2.3	hypothetical protein
TTHA0681	2.5	hypothetical protein
TTHA0682	2.1	hypothetical protein
TTHA0727	5.3	hypothetical protein
TTHA0757	16	hypothetical protein
TTHA1136	4.9	hypothetical protein
TTHA1160	4.2	hypothetical protein
TTHA1626	30	hypothetical protein
TTHA1725	2	hypothetical protein
TTHA1759	2.9	hypothetical protein
TTHA1848	2	hypothetical protein
TTHA1869	2	hypothetical protein
TTHA1870	2.1	hypothetical protein
TTHA1895	4.3	hypothetical protein
TTHA1943	16	hypothetical protein
TTHA1948	2.5	hypothetical protein
TTHA1954	2.7	hypothetical protein
TTHB006	3.6	hypothetical protein
TTHB007	2.9	hypothetical protein
TTHB008	4.3	hypothetical protein
TTHB009	4.8	hypothetical protein
TTHB147	2.4	hypothetical protein
TTHB150	2	hypothetical protein
TTHB156	4.4	hypothetical protein
TTHB157	8.9	hypothetical protein
TTHB158	3.6	hypothetical protein
TTHB203	2.9	hypothetical protein

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**Table 2-3. Cold-inducible genes only in the wild type.**

Function	Gene Name	Discription
Genetic Information Processing Csp2	TTHA0008	phage shock protein A
	TTHA0359	cold shock protein
Metabolism of Carbohydrate	TTHA0022	glucose-1-phosphate adenylyltransferase
	TTHA1261	4-alpha-glucanotransferase (amylomaltase) (disproportionating enzyme) (D-enzyme)
Metabolism of energy	TTHA0021	putative NADPH oxidoreductase
Metabolism of fatty acid	TTHA0750	3-oxoacyl-[acyl carrier protein] reductase
hypothetical protein	TTHA0613	hypothetical protein
	TTHA0777	hypothetical protein
	TTHA0808	hypothetical protein
	TTHA1581	hypothetical protein
	TTHB003	hypothetical protein
	TTHC006	hypothetical protein

**Table 2-4. Cold-repressible genes only in the wild type.**

Function	Gene Name	Discription
transcription	TTHB186	putative transcriptional regulator
transcription	TTHB073	transcriptional regulator
LytR/CspA/Psr family	TTHA1778	LytR/CspA/Psr family protein
Membrane/ Transporter/ Lipoprotein	TTHB076	putative C4-dicarboxylate transporter, large permease protein
	TTHA0452	branched-chain amino acid ABC transporter, ATP-binding protein
	TTHB218	ABC transporter, ATP-binding protein
	TTHA0451	probable branched-chain amino acid ABC transporter, amno acid binding protein
Membrane/ Transporter/ Lipoprotein	TTHA1337	peptide ABC transporter, permease protein
	TTHA1338	ABC transporter permease protein
Metabolism of ammino acid	TTHA0606	aspartate 1-decarboxylase
Metabolism of Carbohydrate	TTHA0506	malate synthase
	TTHA0097	NADH-quinone oxidoreductase chain 14
Metabolism of Cofactors and Vitamins	TTHA0190	dihydroneopterin aldolase
Metabolism of energy	TTHA0663	pyruvate orthophosphate dikinase
	TTHA1276	V-type ATP synthase subunit E
	TTHA1272	V-type ATP synthase subunit B
	TTHA1271	V-type ATP synthase subunit D
	TTHA1273	V-type ATP synthase subunit A
Others /Unknown	TTHA0982	bacterioferritin
	TTHA1335	branched-chain amino acid ABC transporter, ATP-binding protein
hypothetical protein	TTHA0467	hypothetical protein
	TTHA0487	hypothetical protein
	TTHA0646	hypothetical protein
	TTHA0681	hypothetical protein
	TTHA0682	hypothetical protein
	TTHA1160	hypothetical protein
	TTHA1725	hypothetical protein
	TTHA1869	hypothetical protein



**Table 2-5. The genes increased under the cold conditions in *Δtscp1* mutant.**

Function	Gene Name	fold	Description	
Genetic Information Processing	TTHA0841	3.8	stage V sporulation protein R (SpoVR) related protein	
	TTHB016	3.6	oxidoreductase, short-chain dehydrogenase/reductase family	
	TTHA1036	3.4	signal recognition particle protein	
	TTHA1502	3.4	response regulator	
	TTHA1190	2.7	rod shape-determining protein MreD	
	TTHA0249	2.6	preprotein translocase SecE subunit	
	TTHA1002	2.3	response regulator	
	TTHA1189	2.3	rod shape-determining protein MreC	
	TTHA1003	2	sensor histidine kinase	
	Chaperones	TTHA0630	2.8	heat shock protein HslU
		TTHC012	2.7	anti-toxin-like protein
	detoxification	TTHA0554	2.9	small multidrug export protein
		TTHB023	9.3	transcriptional regulator, TetR family
transcription	TTHA0622	6.6	transcription elongation factor GreA	
	TTHA0248	4.4	transcription antitermination protein NusG	
	TTHA1065	3.4	transcription termination factor Rho	
translation	TTHA1679	5.1	30S ribosomal protein S14	
	TTHA1570	2.7	deoxyhypusine synthase	
translation/ ribosome	TTHA1465	2.5	50S ribosomal protein L13	
	TTHA1138	2.1	30S ribosomal protein S15	
	TTHA1819	2.2	2'-5' RNA ligase	
RNA ligase	TTHA0109	3.1	ATP-dependent RNA helicase	
RNA helicase	TTHA1162	3.7	excisionase domain protein	
	TTHA1539	3.4	putative phage integrase/recombinase	
	TTHA1440	3.1	excinuclease ABC subunit A (UvrA)	
	TTHA0112	3.1	endonuclease III	
DNA repair/ recombination/ replication	TTHA0135	7.1	MutT/nudix family protein	
	TTHA1743	4	orotidine 5'-phosphate decarboxylase	
	TTHA1795	2.4	MutT/nudix family protein	
	TTHA0361	2.3	survival protein SurE	
	TTHA1920	3.8	thioredoxin reductase	
Metabolism of nucleotide	TTHA1004	6.8	conserved hypothetical membrane protein	
	TTHA0377	6.1	sugar ABC transporter, permease protein	
	TTHA1391	6	GTP-binding protein	
	TTHA1007	5.6	ABC transporter, ATP-binding protein	
	TTHA1008	5.5	ABC transporter, permease protein	
	TTHA0255	5.1	ferric uptake regulation protein	
	TTHA0725	4.8	membrane-bound protein LytR	
	TTHA1896	3.4	glucosamine--fructose-6-phosphate aminotransferase [isomerizing]	
	TTHB251	3.4	ABC transporter, periplasmic solute-binding protein-related protein	
	TTHA0194	3.3	probable tripartite transporter, small subunit	
	TTHA0627	3.3	large-conductance mechanosensitive channel	
	TTHA1170	3	amino acid ABC transporter, permease protein	
	TTHA1185	2.8	GTP-binding protein	
	TTHA0045	2.5	probable potassium uptake protein TrkA	
	TTHA0629	2.5	putative O-linked GlcNAc transferase (TPR repeat)	
	TTHA1060	2.4	Mg <sup>2+</sup> transporter MgtE	
	TTHA1171	2.4	amino acid ABC transporter, periplasmic amino acid-binding protein	
	TTHA1173	2.2	Trk system potassium uptake protein (TrkG)	
	TTHA0120	2.1	GTP-binding protein Era	
	TTHA0717	2	molybdenum ABC transporter molybdate-binding protein	

Metabolism of amino acid	TTHA1208	2.4	probable citramalate synthase	
	TTHA1457	2.2	S-adenosylmethionine decarboxylase proenzyme	
	TTHA1914	4.9	homocitrate synthase	
	TTHA1844	3.3	anthranilate synthase component I (TrpE)	
	TTHA0760	3.2	O-acetyl-L-homoserine sulphydrylase	
	TTHA1320	3.1	periplasmic serine protease	
	TTHA0759	2.6	homoserine O-acetyltransferase	
	TTHA1911	2.6	probable homoaconitase large subunit (homoaconitate hydratase)	
	TTHA1378	2.5	homoisocitrate dehydrogenase	
	TTHA1642	2.5	S-adenosylmethionine synthetase	
	TTHA0824	2.3	spermidine synthase	
	TTHA0825	2.3	S-adenosylmethionine decarboxylase proenzyme	
	TTHA1910	2.3	probable homoaconitase small subunit (homoaconitate hydratase)	
	TTHA0957	2.2	dihydrodipicolinate synthase	
	TTHA1843	2.2	anthranilate synthase component II (TrpG)	
	TTHA1041	2.1	lysyl-tRNA synthetase (lysine--tRNA ligase) (LysRS)	
	TTHA1842	2	anthranilate phosphoribosyltransferase (TrpD)	
	Metabolism of Carbohydrate	TTHA1066	9	probable transaldolase
		TTHB017	3.5	medium-chain acyl-CoA ligase-related protein
		TTHA1707	2.7	sugar fermentation stimulation protein family protein
TTHA0947		2.4	triosephosphate isomerase	
Metabolism of Cofactors and Vitamins	TTHA0011	2.5	molybdenum cofactor biosynthesis protein A (MoaA)	
	TTHA0929	2.2	phosphopantetheine adenylyltransferase	
	TTHA1796	2	probable thiamine biosynthesis protein ThiI	
Metabolism of energy	TTHA1326	5	cytochrome c-552 like protein	
	TTHA1325	4.7	putative sulfite oxidase	
	TTHA1422	2.3	thioredoxin	
Metabolism of fatty acid	TTHB022	9.8	putative acyl-CoA dehydrogenase	
	TTHB020	5.9	3-oxoacyl-[acyl carrier protein] reductase	
	TTHB015	5.7	putative acyl-CoA dehydrogenase	
	TTHA0948	2.8	fatty acid desaturase	
Others /Unknown	TTHA0962	6.8	homoprotocatechuate 2,3-dioxygenase	
	TTHA0840	3.3	probable thiol:disulfide interchange protein	
	TTHA0838	2.9	glutaredoxin-like protein	
	TTHA1140	2.4	metallo-beta-lactamase superfamily protein	
	TTHA0737	2	putative dihydrodipicolinate synthase	
	TTHB014	15	probable phosphotransferase	
	TTHB012	9.3	phosphoglycerate mutase family protein	
	TTHB117	5.4	putative type IV pilin	
	TTHA1511	4	putative catechol 1,2-dioxygenase	
	TTHB119	4	prepilin-like protein	
	TTHA1209	3.2	probable acetyltransferase	
	TTHA1619	2.5	probable methyltransferase	
	TTHA1512	2.4	putative nucleotidyltransferase	
	TTHA1897	2.4	GidA-related protein	
	TTHA1217	2.2	prepilin-like protein	
	TTHB201	2.2	transposase-like protein	
	TTHA0241	3.1	oxidoreductase, short-chain dehydrogenase/reductase family	
	TTHA0013	2.2	geranylgeranyl diphosphate synthetase	

hypothetical protein

TTHA0025	2.2	hypothetical protein
TTHA0079	2.9	hypothetical protein
TTHA0105	2.4	hypothetical protein
TTHA0111	3.6	hypothetical protein
TTHA0163	5.5	hypothetical protein
TTHA0212	5	hypothetical protein
TTHA0228	2	hypothetical protein
TTHA0253	2.5	hypothetical protein
TTHA0362	2.2	hypothetical protein
TTHA0396	2.7	hypothetical protein
TTHA0419	2.1	hypothetical protein
TTHA0539	2.3	hypothetical protein
TTHA0560	2	hypothetical protein
TTHA0568	4.4	hypothetical protein
TTHA0592	2.8	hypothetical protein
TTHA0702	3.1	hypothetical protein
TTHA0728	2.9	hypothetical protein
TTHA0802	2	hypothetical protein
TTHA0828	2.8	hypothetical protein
TTHA0942	2.3	hypothetical protein
TTHA0961	3.5	hypothetical protein
TTHA1001	4.7	hypothetical protein
TTHA1005	6	hypothetical protein
TTHA1006	6.6	hypothetical protein
TTHA1075	2.1	hypothetical protein
TTHA1102	2.4	hypothetical protein
TTHA1108	2.2	hypothetical protein
TTHA1118	2.5	hypothetical protein
TTHA1158	3.3	hypothetical protein
TTHA1175	2.9	hypothetical protein
TTHA1187	2.1	hypothetical protein
TTHA1268	2.3	hypothetical protein
TTHA1327	4.4	hypothetical protein
TTHA1467	2	hypothetical protein
TTHA1510	3	hypothetical protein
TTHA1708	2.4	hypothetical protein

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**Table 2-6. The genes decreased under the cold conditions in *Δttcsp1* mutant.**

Function	Gene Name	fold	Discription
Genetic Information Processing	TTHA0829	5.2	putative acetoin utilization protein, acetoin dehydrogenase
	TTHA1774	2.6	pili retraction protein PilT
	TTHA1352	2.1	response regulator
	TTHA1625	2.1	osmotically inducible protein OsmC
Chaperones	TTHA1499	6.8	MoxR-related protein
	TTHA1487	2.1	ATP-dependent Clp protease, ATP-binding subunit ClpB
transcription	TTHA0058	2.52	transcriptional repressor of class I heat-shock genes
	TTHB248	2.3	transcriptional regulator, IclR family
	TTHA0508	2	transcriptional regulator, MerR family
translation	TTHA1498	9.5	elongation factor G (EF-G-2)
DNA repair/ recombination/ replication	TTHA1806	2.1	MutM protein (formamidopyrimidine-DNA glycosylase) (Fpg)
Metabolism of nucleotide	TTHA0735	3.1	cytidine deaminase
Membrane/ Transporter/ Lipoprotein	TTHA1141	6.5	cation-transporting ATPase
	TTHA1137	4.5	major facilitator superfamily transporter
	TTHA1407	4.1	cytochrome c-type biogenesis protein, heme exporter protein B
	TTHA1408	3.9	ABC transporter, ATP-binding protein
	TTHA1651	3	maltose ABC transporter, permease protein
	TTHA1652	2.6	maltose ABC transporter, periplasmic maltose-binding protein
	TTHA0566	2.4	GTP-binding protein HflX
	TTHA1807	2.4	ABC transporter, permease protein
	TTHA0766	2.3	ABC transporter, solute-binding protein
	TTHA0767	2.3	putative small integral membrane tranport protein
	TTHA0977	2.2	sugar ABC transporter, permease protein
	TTHA0356	2	ABC transporter, periplasmic substrate-binding protein
	Metabolism of amino acid	TTHA0237	2
TTHA1246		2.5	methylmalonyl-CoA mutase
TTHA0489		2.3	homoserine dehydrogenase
TTHA0500		2.3	glycerate dehydrogenase/hydroxypyruvate reductase
TTHA0534		2.2	aspartokinase (aspartate kinase) [contains: aspartokinase alpha subunit; aspartokinase beta subunit]
TTHA0525		2	glycine dehydrogenase (decarboxylating) subunit 1
Metabolism of Carbohydrate	TTHA1836	27	isocitrate lyase
	TTHA0233	3.2	pyruvate dehydrogenase complex, dihydroliipoamide dehydrogenase E3 component
	TTHA0278	3.2	ATP-dependent phosphoenolpyruvate carboxykinase
	TTHA0232	3	pyruvate dehydrogenase complex, dihydroliipoamide acetyltransferase E2 component
	TTHA0595	2.4	galactokinase
	TTHA0726	2.4	aconitate hydratase (aconitase)
	TTHA1809	2.4	proline iminopeptidase-related protein
	TTHA1113	2.1	L-lactate dehydrogenase
	Metabolism of Cofactors and Vitamins	TTHA1506	3.3
TTHA1775		2.6	pantoate--beta-alanine ligase
TTHA0206		2.4	nicotinamide nucleotide transhydrogenase, alpha subunit 1

Metabolism of energy	TTHA1134	32	ba3-type cytochrome c oxidase polypeptide II	
	TTHA1135	23	ba3-type cytochrome c oxidase polypeptide I	
	TTHA1133	22	ba3-type cytochrome c oxidase polypeptide IIA	
	TTHA1447	11	alanine dehydrogenase	
	TTHA1942	11	putative cytochrome c oxidase assembly protein	
	TTHA1500	7.7	phosphoenolpyruvate synthase	
	TTHA1505	3.4	cytochrome c assembly protein-related protein	
	TTHA0486	2.8	probable iron-sulfur protein	
	TTHA1117	2.7	iron-sulfur protein	
	TTHA1930	2.7	quinol-cytochrome c reductase, cytochrome b subunit	
	TTHA0310	2.5	cytochrome c oxidase assembly factor (CtaA) + protoheme IX farnesyltransferase (CtaB)	
	TTHA0089	2.33	NADH-quinone oxidoreductase chain I	
	TTHA0152	2.02	tungsten-containing aldehyde:ferredoxin oxidoreductase	
	TTHA0153	2.01	NADPH:quinone reductase	
	TTHA1933	2	probable c-type cytochrome	
	Metabolism of fatty acid	TTHA1463	10	long-chain-fatty-acid--CoA ligase
		TTHB116	2.5	acyl-CoA dehydrogenase, short-chain specific (probable AidB protein)
		TTHA0604	2.1	medium-chain-fatty-acid--CoA ligase
	Others /Unknown	TTHA0642	3.2	putative glycosyltransferase
		TTHA0632	2	predicted nucleotidyltransferase
TTHA1448		8.5	4-hydroxybutyrate CoA-transferase	
TTHB010		3.6	transposase-related protein	
TTHB074		3.1	putative C4-dicarboxylate transporter, periplasmic C4-dicarboxylate-binding protein	
TTHA1317		2.8	immunogenic protein related protein	
TTHA1259		2.3	adenylate cyclase related protein	
TTHA1612		2.1	putative hydrolase	
TTHA0488		2.5	antitoxin of toxin-antitoxin stability system	
hypothetical protein	TTHA1626	34	hypothetical protein	
	TTHA0757	17	hypothetical protein	
	TTHA1943	15	hypothetical protein	
	TTHB009	13	hypothetical protein	
	TTHA0316	11	hypothetical protein	
	TTHB203	8.6	hypothetical protein	
	TTHB008	8.1	hypothetical protein	
	TTHA0727	6.6	hypothetical protein	
	TTHB156	6.4	hypothetical protein	
	TTHB006	5.4	hypothetical protein	
	TTHA1759	5.4	hypothetical protein	
	TTHB158	5	hypothetical protein	
	TTHB204	4.6	hypothetical protein	
	TTHB157	4.3	hypothetical protein	
	TTHA1895	4.2	hypothetical protein	
	TTHA1954	3.9	hypothetical protein	
	TTHA1837	3.9	hypothetical protein	
	TTHA1752	3.7	hypothetical protein	
	TTHA1136	3.7	hypothetical protein	
	TTHB007	3.6	hypothetical protein	
	TTHA0463	3.3	hypothetical protein	
	TTHB147	3.2	hypothetical protein	

TTHA0641	3.2	hypothetical protein
TTHB150	3.1	hypothetical protein
TTHA0332	3.1	hypothetical protein
TTHA0980	2.9	hypothetical protein
TTHB185	2.6	hypothetical protein
TTHB149	2.6	hypothetical protein
TTHB151	2.6	hypothetical protein
TTHA1301	2.6	hypothetical protein
TTHA0168	2.53	hypothetical protein
TTHB148	2.5	hypothetical protein
TTHA1247	2.5	hypothetical protein
TTHA0603	2.5	hypothetical protein
TTHA1870	2.4	hypothetical protein
TTHA0640	2.3	hypothetical protein
TTHA0501	2.3	hypothetical protein
TTHA0780	2.3	hypothetical protein
TTHA1610	2.3	hypothetical protein
TTHB152	2.2	hypothetical protein
TTHA1020	2.2	hypothetical protein
TTHA1848	2.1	hypothetical protein
TTHB165	2.1	hypothetical protein
TTHA0268	2.1	hypothetical protein
TTHA0236	2.1	hypothetical protein
TTHA1948	2.1	hypothetical protein
TTHA1258	2	hypothetical protein
TTHA0464	2	hypothetical protein
TTHB126	2	hypothetical protein
TTHA1045	2	hypothetical protein
TTHA1646	2	hypothetical protein

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### 3. Proteome analysis

To know more substantive effects of the transcriptional changes on the cells under cold stress condition, I performed proteome analysis of the extracts from the wild type and *Δttcsp1* mutant, using 2D-PAGE and MALDI-TOF-MS. For the proteome analysis of the wild type under cold stress condition, the cells were grown until the absorbance of culture at 600 nm reached 0.8 before transferring the culture from 70°C to 45°C. As a first step, the whole cell lysates of each cell grown at 70°C and 45°C were separated by using pH 3 to 10 broad range strip followed by the second dimension on 12.5% linear SDS-PAGE gel. However, I could not distinguish up- and down-regulated proteins under cold stress condition due to abundant proteins in whole cell lysates (data not shown). Therefore, I carried out fractionation of the whole cell lysate into soluble fraction (cytosolic proteins) and insoluble fraction (membrane and cell wall proteins) using ultracentrifugation at 105,000 g for 1 h before 2-DE separation. After ultracentrifugation, the soluble and insoluble proteins were resolved onto pH 4 to 7 strip in the first dimension and 12.5% SDS-PAGE 2-DE gel in the second dimension. This procedure enabled us to detect changes in protein spots on gels. According to this procedure, the samples of the wild type and *Δttcsp1* mutant strains were prepared after transfer of the cultures from 70°C to 45°C and further incubation at 45°C for 30 min. This cold shock treatment was likely to confer acclimating period before cold adaptation to cells. Totally, eight sets of 2-DE gel, cytosolic and membrane proteins for the wild type and *Δttcsp1* mutant grown under optimal and cold stress conditions, respectively, were prepared. The analysis of 2-DE gels by ImageMaster Premium 5.0 allowed spot

detection, pairing and matching of each group of 2-DE gel. The 2-DE map for cytosolic proteins was completely different from that of membrane proteins, indicating successful separation of soluble cytosolic proteins and insoluble membrane proteins by ultracentrifugation. This improved procedure is a simple and valuable approach method for 2-DE based proteome study in *T. thermophilus* HB8.

Table 3-1 represents respectively detected spot numbers on the 2-DE gel of the eight samples, which was named G-III to G-X as a group. More than 740 and 240 protein spots were detected at cytosolic and membrane protein samples on 2-DE gel, respectively. Tables 3-2 and 3-3 represent the percentage of gel-to-gel matching of spots in cytosolic and membrane proteins of each group by ImageMaster, respectively. Spot matching across the obtained sets of gel images showed more than 50%. These spots could be used to compare expression level between respective groups. The quantitative change of concerned spots was carefully evaluated using triple different gels for each group sample. Significant changes ( $p < 0.05$ ) of up- or down-regulated protein spots between 2-DE data were tested by Student's *t*-test to eliminate false positive and false negative results.



**Table 3-1. Detected spot number on the 2-DE gel of each group.**

Group name	Sample name	Spots*
G-III	wild type, 70°C, 30 min, cytosolic protein	983.3 ± 97
G-IV	<i>Δttcsp1</i> , 70°C, 30 min, cytosolic protein	1034.3 ± 31
G-V	wild type, 45°C, 30 min, cytosolic protein	896.0 ± 66
G-VI	<i>Δttcsp1</i> , 45°C, 30 min, cytosolic protein	742.0 ± 14
G-VII	wild type, 70°C, 30 min, membrane protein	253.3 ± 18
G-VIII	<i>Δttcsp1</i> , 70°C, 30 min, membrane protein	240.0 ± 53
G-IX	wild type, 45°C, 30 min, membrane protein	290.3 ± 52
G-X	<i>Δttcsp1</i> , 45°C, 30 min, membrane protein	248.6 ± 8

\*The results represent detected spot number from different triple 2-DE gels with standard deviation.

**Table 3-2. Spot matching across gel images for cytosolic proteins of each group.**

	Pair matches result of cytosolic proteins (%)*			
	G-III	G-IV	G-V	G-VI
G-III	67.3 ± 4.2 <sup>†</sup>	67.6 ± 5.1	69.4 ± 1.4	ND <sup>#</sup>
G-IV		79.6 ± 1.1	ND <sup>#</sup>	67.4 ± 2.7
G-V			84.7 ± 0.8	74.5 ± 1.2
G-VI				84.6 ± 1.2

\*The results represent percentage of spot matching of each group with cognate group.

<sup>†</sup> The matching results were derived from different triple 2-DE gels with standard deviation.

<sup>#</sup> ND, not determined.

**Table 3-3. Spot matching across gel images for membrane proteins of each group.**

	Pair match result of membrane proteins (%)*			
	G-VII	G-VIII	G-IX	G-X
G-VII	78.9 ± 1.6 <sup>†</sup>	74.1 ± 1.9	51.8 ± 3.3	ND <sup>#</sup>
G-VIII		82.3 ± 1.2	ND <sup>#</sup>	60.0 ± 3.8
G-IX			73.5 ± 4.7	62.0 ± 19
G-X				78.1 ± 0.4

\*The results represent percentage of spot matching of each group with cognate group.

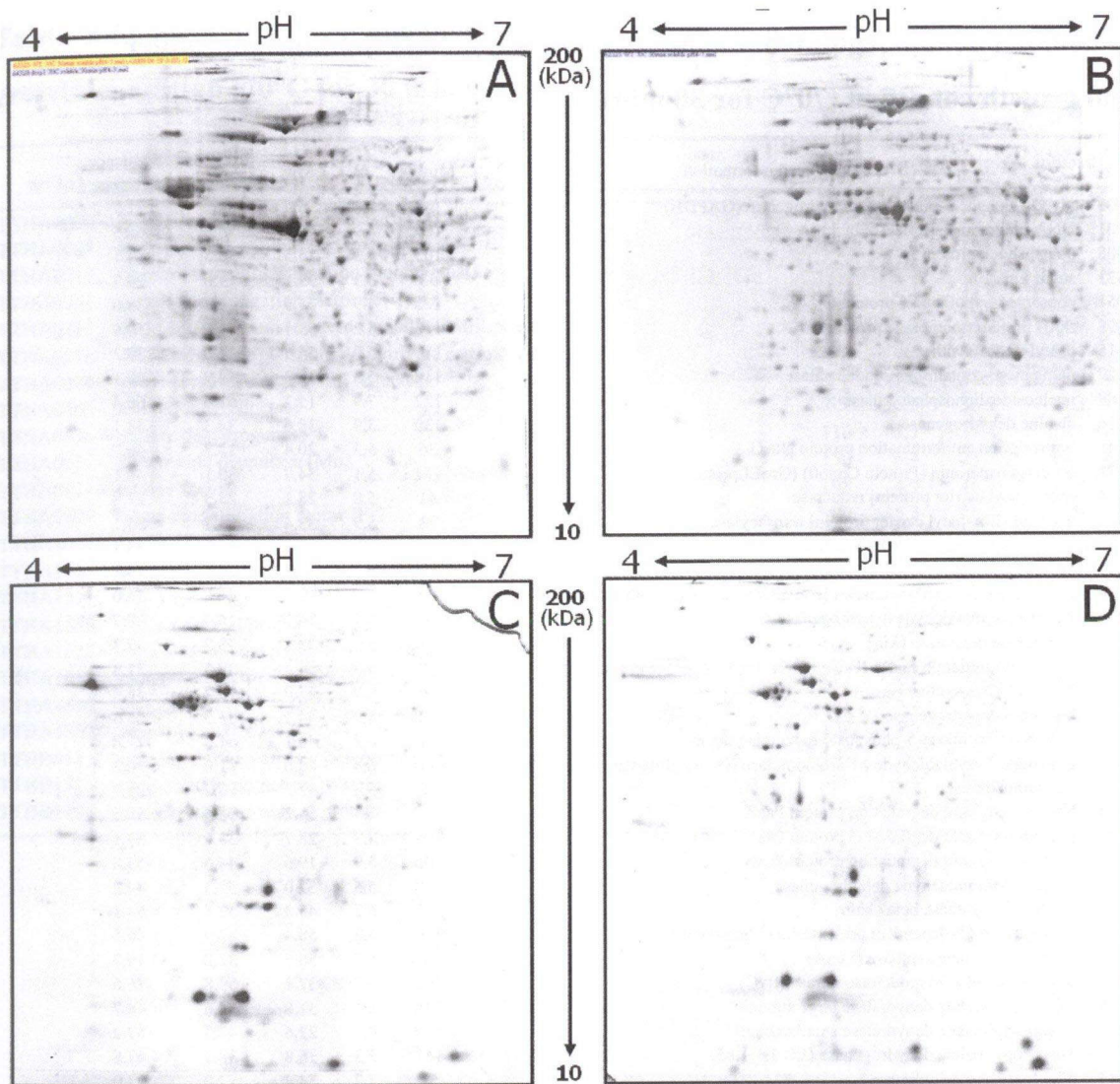
<sup>†</sup> The matching results were derived from different triple 2-DE gels with standard deviation.

<sup>#</sup> ND, not determined.

### 3-1 Difference of proteome results between the wild type and $\Delta ttcsp1$ mutant under optimal growth condition.

To investigate the functional role of *tCsp1*, I first focused on proteins that showed different expression pattern between the wild type and  $\Delta ttcsp1$  mutant strains under optimal growth condition (at 70°C). Figure 3-1 showed 2-DE maps of cytosolic and membrane proteins of the wild type (A and C) and  $\Delta ttcsp1$  mutant (B and D) on the pH 4 to 7 and 12.5% SDS-PAGE 2-DE gel. The gel-to-gel matching of spots (wild type vs. the  $\Delta ttcsp1$  mutant) showed 67.6% (G-III vs. G-IV) and 74.1% (G-VII vs. G-VIII) for cytosolic and membrane protein fractions, respectively (Tables 3-2 and 3-3). By comparing these spots between two strains, I identified up- and down-regulated proteins in the  $\Delta ttcsp1$  mutant grown at 70°C against the wild type grown at the same temperature. In cytosolic protein fraction, 63 up-regulated (Table 3-4) and 23 down-regulated (Table 3-5) proteins showed more than 1.5 times expression change and  $p < 0.05$ . In membrane protein fraction, 8 up-regulated (Table 3-6) and 9 down-regulated (Table 3-7) proteins were identified on the same criteria.

In the cytosolic protein fraction, some translational controllers including translation elongation factor EF-Tu (both TTHA0251 and TTHA1694), translation initiation factor IF-2 (TTHA0699) and elongation factor EF-G (TTHA1695) were down-regulated at 70°C in the  $\Delta ttcsp1$  mutant against the wild type (Table 3-5). It implies that *tCsp1* has influences on translational control. On the other hand, as shown in Table 3-4, many essential factors for cell growth are down-regulated by *tCsp1*. *tCsp1* may ready to stall cell growth in case of some stresses.



**Figure 3-1. 2-DE proteome profiling of the wild type and  $\Delta ttcsp1$  mutant under 70°C after 30 min from transient state.**

The cytosolic and membrane proteins were extracted from wild type and  $\Delta ttcsp1$  mutant after transfer to 70°C for 30 min. Each protein sample was separated onto the pH 4 to 7, and 12.5% SDS-PAGE gel (13 cm x 15 cm x 1 mm) and followed CBB G-250 staining with aluminium sulfate. **A**, The cytosolic proteins of the wild type. **B**, The cytosolic proteins of  $\Delta ttcsp1$  mutant. **C**, The membrane proteins of the wild type. **D**, The membrane proteins of  $\Delta ttcsp1$  mutant.

**Table 3-4. The up-regulated cytosolic proteins in *Δtscp1* mutant under optimal growth condition (70°C for 30 min).**

orf ID	Annotated information	Mowse	pI	Mw.	Intensity coverage	Sequence coverage
TTHA0905	glyceraldehyde 3-phosphate dehydrogenase(GAPDH)	74	7.8	35.9	30.5	31.7
TTHA0001	DNA polymerase III beta subunit	167	4.9	40.5	34.6	60.5
TTHA0008	phage shock protein A	103	5.3	25.5	30.5	54.1
TTHA0431	sugar kinase	53	5.3	32.1	19.0	29.7
TTHA0051	conserved hypothetical protein	125	4.9	31.5	42.7	38.0
TTHA0098	SufB protein (membrane protein)	173	5.2	53.1	41.8	50.9
TTHA0115	prolyl-tRNA synthetase	117	5.9	54.5	44.0	38.2
TTHA0122	manganese-containing pseudocatalase	112	5.3	33.3	44.9	40.1
TTHA0188	nucleoside diphosphate kinase	122	7.9	15.3	61.6	66.4
TTHA0216	alanine dehydrogenase	130	5.9	38.6	34.3	53.1
TTHA0248	transcription antitermination protein NusG	86	5.3	20.4	17.3	46.7
TTHA0271	60 kDa chaperonin (Protein Cpn60) (GroELprotein)	171	5.1	54.7	50.1	44.1
TTHA0304	enoyl-[acyl carrier protein] reductase	51	5.8	28.1	23.3	21.8
TTHA0416	malonyl CoA-[acyl carrier protein] transacylase	78	5.9	33.4	34.1	35.1
TTHA0432	IMP dehydrogenase/GMP reductase	96	6.5	53.1	40.6	33.1
TTHA0506	malate synthase	69	6.1	58.7	20.6	19.1
TTHA0534	aspartokinase (aspartate kinase) [contains:aspartokinase alpha subun	134	5.7	43.3	44.0	36.0
TTHA0545	aspartate-semialdehyde dehydrogenase	86	6.1	36.1	16.0	38.7
TTHA0557	superoxide dismutase [Mn]	71	6.4	23.2	38.2	37.3
TTHA0699	translation initiation factor IF-2	88	5.3	56.4	39.5	31.8
TTHA0712	histidyl-tRNA synthetase	76	6.0	47.0	18.2	35.4
TTHA0722	histidinol dehydrogenase	230	4.9	44.1	51.5	65.5
TTHA0856	1-deoxy-D-xylulose-5-phosphate reductoisomerase	81	6.2	40.1	41.5	27.8
TTHA0934	glutamate-1-semialdehyde 21-aminomutase (GSA)(glutamate-1-semialdeh	140	6.4	46.0	81.0	30.2
TTHA0940	aminotransferase	52	5.8	42.2	10.2	25.7
TTHA0968	phenylacetic acid degradation protein PaaZ	158	6.4	55.2	48.4	28.5
TTHA0970	phenylacetic acid degradation protein PaaC	106	4.7	28.7	38.9	37.1
TTHA0971	phenylacetic acid degradation protein PaaB	106	5.9	19.6	44.6	53.8
TTHA0996	succinate-semialdehyde dehydrogenase	139	5.5	52.0	52.3	44.2
TTHA1095	tryptophan synthase beta chain	182	6.2	45.3	52.8	54.1
TTHA1111,	alternative ATP-dependent protease La (Lonprotease)	157	5.2	58.4	42.9	46.5
TTHA1210	2-isopropylmalate synthase (LeuA)	60	5.7	56.5	32.2	19.7
TTHA1211,	probable ketol-acid reductoisomerase (IlvC)	242	6.2	37.1	59.8	76.6
TTHA1228,	3-isopropylmalate dehydratase large subunit	148	6.4	51.8	54.6	48.7
TTHA1229	3-isopropylmalate dehydratase small subunit	152	4.7	22.6	47.3	57.2
TTHA1230	3-isopropylmalate dehydrogenase (EC 1.1.1.85)	141	5.3	36.8	38.1	47.8
TTHA1234	dihydroxyacid dehydratase	52	5.7	54.4	17.0	23.0
TTHA1243	septum site-determining protein MinD	172	5.3	28.9	58.2	55.4
TTHA1248	acetyl-coenzyme A synthetase	158	6.0	58.4	38.9	38.1
TTHA1329	glutamine synthetase	99	6.2	50.5	46.3	39.7
TTHA1355,	DNA gyrase subunit A	79	6.0	57.0	38.7	20.1
TTHA1392	Sir2 family protein	60	5.7	27.8	9.7	22.4
TTHA1435	purine nucleoside phosphorylase	118	6.1	25.4	47.4	51.9
TTHA1498	elongation factor G (EF-G-2)	87	5.1	56.7	24.2	29.5
TTHA1519	phosphoribosylformylglycinamide synthase II	154	5.5	55.7	26.5	45.5
TTHA1535	isocitrate dehydrogenase	91	6.3	54.4	35.0	24.4
TTHA1557	propionyl-CoA carboxylase beta subunit	78	5.8	55.9	38.2	26.6
TTHA1625	osmotically inducible protein OsmC	115	5.4	15.3	63.1	66.9
TTHA1649	nucleotidyltransferase	52	7.8	10.6	25.6	47.4
TTHA1659	tetratricopeptide repeat family protein	184	6.1	48.9	56.4	38.6
TTHA1664	DNA-directed RNA polymerase alpha chain	214	4.8	35.0	58.7	57.8
TTHA1695	elongation factor G (EF-G)	120	5.2	57.2	22.9	43.9
TTHA1698	carboxypeptidase G2	72	5.8	40.0	17.2	23.0
TTHA1797,	probable amidase	73	5.9	46.5	15.1	27.4
TTHA1814	SufD protein (membrane protein)	75	6.1	47.9	15.3	30.9
TTHA1816	rod shape-determining protein MreB	106	5.4	36.8	54.3	29.0
TTHA1836	isocitrate lyase	55	5.6	49.1	12.2	22.1
TTHA1840	SufD protein (membrane protein)	214	6.1	47.9	44.6	62.9
TTHA1914	homocitrate synthase	216	5.8	42.1	58.2	62.2
TTHA0987	beta-ketoadipyl CoA thiolase	89	6.0	42.7	44.7	31.2
TTHB125	chromosome partitioning ATPase ParA family	111	6.6	35.9	55.8	32.6
TTHB152	conserved hypothetical protein	185	6.8	51.1	60.9	42.2
TTHB190	conserved hypothetical protein	290	5.6	40.8	80.8	66.8

**Table 3-5. The down-regulated cytosolic proteins in *Δttcsp1* mutant under optimal growth condition (70°C for 30 min).**

orf I.D	Annotated information	Mowse	pI	Mw.	Intensity coverage	Sequence coverage
TTHA0090	NADH-quinone oxidoreductase chain 3	109	5.6	57.1	40.9	21.1
TTHA0098	arginyl-tRNA synthetase	177	6.6	57.5	65.5	43.6
TTHA0248,	transcription antitermination protein NusG	51	5.3	20.4	21.7	32.6
TTHA0251	translation elongation factor EF-Tu.B	50	5.3	44.8	14.4	32.5
TTHA0271	60 kDa chaperonin (Protein Cpn60) (GroEL protein)	197	5.1	54.7	49.9	42.6
TTHA0278	ATP-dependent phosphoenolpyruvate carboxykinase	172	6.4	57.6	45.4	47.5
TTHA0304	enoyl-[acyl carrier protein] reductase	54	5.8	28.1	31.0	25.3
TTHA0465	thioredoxin reductase	65	6.5	36.2	15.3	39.1
TTHA0536	malate dehydrogenase	116	5.7	35.4	35.6	52.3
TTHA0557	superoxide dismutase [Mn]	150	6.4	23.2	54.6	54.4
TTHA0614	trigger factor	198	4.9	46.3	55.4	43.1
TTHA0699	translation initiation factor IF-2	48	5.3	56.4	18.4	31.4
TTHA1066	probable transaldolase	113	5.5	24.0	68.7	45.7
TTHA1123	acetyl-CoA carboxylase biotin carboxylase subunit	208	6.1	49.3	57.5	51.2
TTHA1355	DNA gyrase subunit A	114	6.0	57.0	33.4	35.0
TTHA1589	50S ribosomal protein L25 (TL5)	142	5.1	23.2	70.3	42.7
TTHA1637	ribose-phosphate pyrophosphokinase	106	5.9	33.5	60.5	46.1
TTHA1694	elongation factor Tu (EF-Tu)	257	5.3	44.8	56.4	67.2
TTHA1695	elongation factor G (EF-G)	90	5.2	57.2	27.1	25.4
TTHA1852	oligoendopeptidase F	132	5.5	58.0	46.8	32.0
TTHB052	cobalamin biosynthesis precorrin-8X isomerase	147	7.8	23.2	44.5	52.8
TTHB152	conserved hypothetical protein	60	6.8	51.1	13.9	24.1
TTHB179	conserved hypothetical protein	59	9.8	23.9	9.4	37.6

**Table 3-6. The up-regulated membrane proteins in *Δttcsp1* mutant under optimal growth condition (70°C for 30 min).**

orf I.D	Annotated information	Mowse	pI	Mw.	Intensity coverage	Sequence coverage
TTHA0075	ribonucleoside-diphosphate reductase	95	6.0	57.2	26.3	29.5
TTHA0271	60 kDa chaperonin (Protein Cpn60) (GroELprotein)	233	5.1	54.7		
TTHA0359	cold shock protein					
TTHA0509	N-acyl-L-amino acid amidohydrolase		5.5	47.9		
TTHA0525	glycine dehydrogenase (decarboxylating) subunit 1					
TTHA1210	2-isopropylmalate synthase (LeuA)	56	5.7	56.5		
TTHA1689	50S ribosomal protein L2	201	11.7	30.4		
TTHA1813	DNA-directed RNA polymerase beta chain (RpoB)	147	6.3	57.8		

**Table 3-7. The down-regulated membrane proteins in *Δttcsp1* mutant under optimal growth condition (70°C for 30 min).**

orf I.D	Annotated information	Mowse	pI	Mw.	Intensity coverage	Sequence coverage
TTHA0206	nicotinamide nucleotide transhydrogenase, alpha subunit 1					
TTHA0229	2-oxoisovalerate dehydrogenase E1 componentalpha subunit	141	5.3	41.1		
TTHA0246	50S ribosomal protein L1					
TTHA1272	V-type ATP synthase subunit B					
TTHA1483	conserved putative protein	123	5.9	25.7		
TTHA1487	ATP-dependent Clp protease, ATP-binding subunit ClpB					
TTHA1570	deoxyhypusine synthase					
TTHA1642	S-adenosylmethionine synthetase					
TTHA1818	RecA protein	189	5.5	35.4		

### 3-2 Cold stress response of the wild type under 45°C

Next, I investigated the response of the wild-type to cold stress condition. Figure 3-2 shows 2-DE proteome profiling of the wild type under 70°C and 45°C. In the cytosolic protein fraction, PMF analysis using MALDI-TOF MS identified 34 up-regulated (Table 3-8) and 24 down-regulated protein (Table 3-9) spots at 45°C which showed more than 1.5 times expression change by comparing G-III to G-V data. In addition, 43 up-regulated protein spots (Table 3-10) and 19 down-regulated proteins (Table 3-11) at 45°C were also identified in membrane protein samples. In the following, I describe the observed changes by classifying proteins into their functional categories.

**(i) Translation process.** A lot of up-regulated and highly expressed proteins under cold stress condition are involved in translation process: TTHA0162, 30S ribosomal proteins S1; TTHA0251, translation elongation factor EF-Tu; TTHA0271, 60 kDa chaperonin; TTHA0272, 10 kDa chaperonin; TTHA0699, translation initiation factor IF-2; TTHA0614, trigger factor TTHA0098, arginyl-tRNA synthetase; and TTHA0573, glutamyl-tRNA amidotransferase subunit A. These results suggest that the up-regulation of these proteins rescues impaired translation process significantly under cold stress condition. mRNA-stabilizing proteins and translation related proteins have been defined as up-regulated proteins under cold shock condition for various bacteria. It was reported that various bacteria overexpressed the following proteins as a cold shock response protein: 60kDa chaperonin, 10 kDa chaperonin, cold shock protein (TTHA0359), EF-Tu, NusA, 30S ribosomal protein S1, S-adenosyl methionine synthetase (TTHA1642), trigger factor, RNA polymerase  $\alpha$  chain (TTHA1664), and RNA polymerase  $\beta$  chain (TTHA1813).

**(ii) Amino acid metabolism.** Several proteins involved in amino acid metabolism were up-regulated under cold stress condition: TTHA1577, putative NAD-dependent glutamate dehydrogenase; TTHA0124, branched-chain amino acid aminotransferase; TTHA1329, glutamine synthetase; and with TTHA0525, glycine dehydrogenase.

**(iii) Proteases.** I also found up-regulation of peptidase family proteins at 45°C: TTHA0770, ATP-dependent protease La; TTHA1111, alternative ATP-dependent protease La; TTHA1487, ATP-dependent Clp protease ATP-binding subunit ClpB; and TTHA0256, leucine aminopeptidase.

**(vi) Cold shock proteins.** *ttCsp2* (TTHA0359) was dramatically up-regulated in the wild type cells under 45°C, demonstrating that *ttCsp2* plays important role under cold stress condition. The difference of *ttCsp1* (TTHA0175) expression under between cold stress condition and optimal growth condition was not detected onto pH 3-10 broad range strip and 16.5% SDS-PAGE-Tricine gels. However, the *ttCsp1* was constitutively expressed under both 70°C and 45°C using 15% Tris-Tricine SDS-PAGE gel and western blotting (data not shown). This result was in agreement with the result of transcriptome analysis.

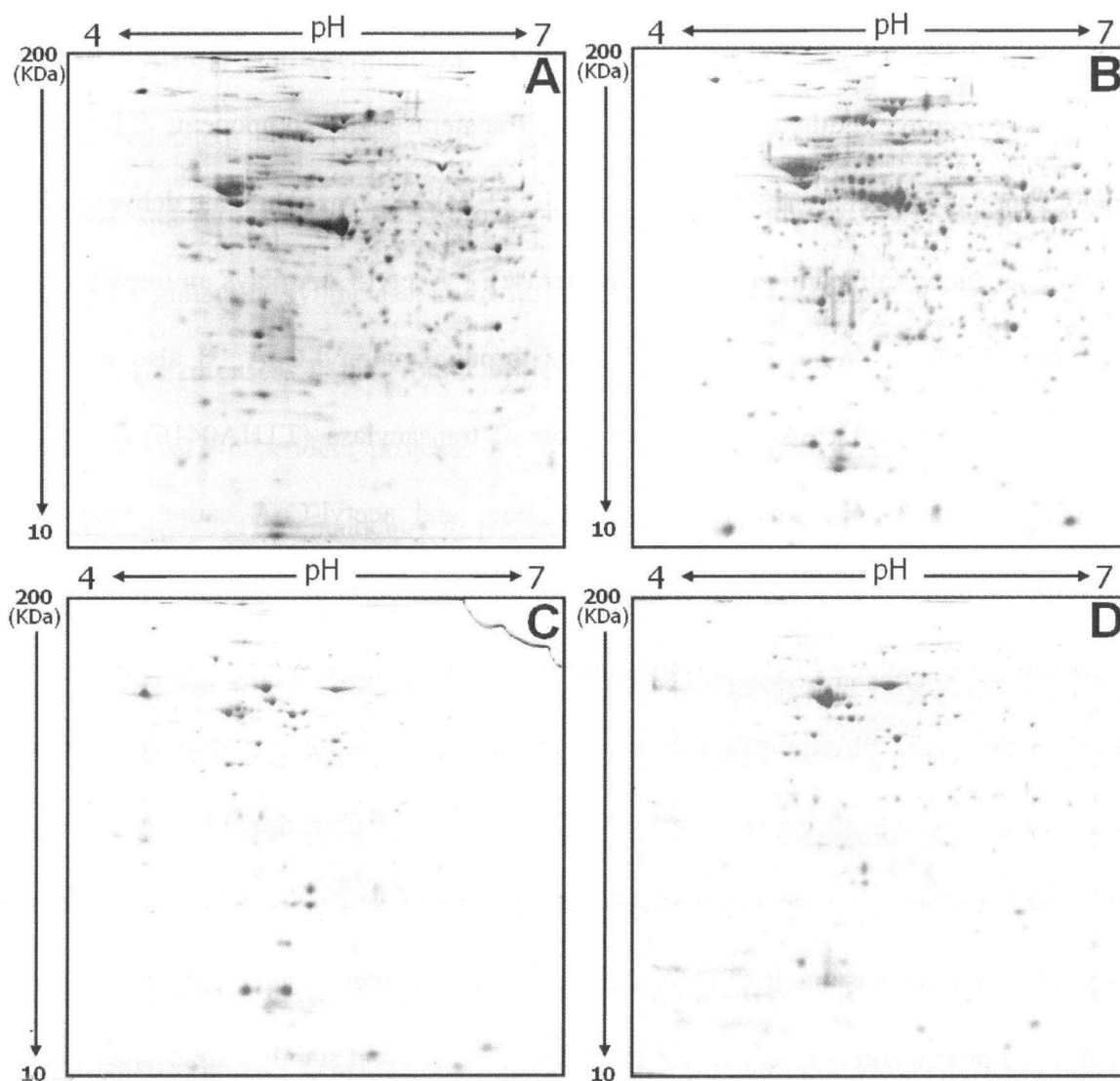
**(v) Energy production.** Several subunits of V-type ATP synthase were severely down-regulated for 30 min under cold stress condition: TTHA1272, ATP synthase subunit B; TTHA1273, ATP synthase subunit A; TTHA1276, ATP synthase subunit E; and TTHA1279, ATP synthase subunit V. This result suggests that energy production is reduced during acclimation period under cold stress condition.

**(vi) Fatty acid synthesis.** The wild type cells increased expression of several proteins associated with acetyl-CoA metabolism under cold stress condition: TTHA0229,



2-oxoisovalerate dehydrogenase E1 component  $\alpha$  subunit; TTHA0232, pyruvate dehydrogenase complex dihydrolipoamide acetyltransferase E2 component; TTHA0287, 2-oxoglutarate dehydrogenase E3 component; and TTHA0288, 2-oxoglutarate dehydrogenase E2 component (dihydrolipoamide succinyltransferase). Acetyl-CoA plays an important role as a building block in production of fatty acids through malonyl-CoA. I also found the up-regulation of malonyl CoA-[acyl carrier protein] transacylase (TTHA0416) and citrate synthase (TTHA1343) in cytosolic protein fraction, and acetyl-CoA carboxylase biotin carboxylase subunit (TTHA1123), propionyl-CoA carboxylase  $\alpha$  subunit (TTHA1148), and 2-siopropylmalate synthase (TTHA1210) in membrane protein fraction. These proteins are involved in fatty acid biosynthesis pathway. In contrast, I found the down-regulation of proteins which are able to degrade acetyl-CoA and malonyl CoA: acetyl-CoA synthetase (TTHA1248), malate synthase (TTHA0506), 3-isopropylmalate dehydratase large subunit (TTHA1228) and small subunit (TTHA1229). These proteome results support the notion that fatty acid production is effectively activated to maintain and stabilize lipid bilayer under cold stress condition.

As indicated above (i) to (vi), components of the cell are drastically changed. Some of these alteration may result from the function of *tCsp1* and others may not. To investigate which changes were caused by *tCsp1*, the influences of deletion of *ttcsp1* on the transcriptome analysis were determined in the following section.



**Figure 3-2. 2-DE proteome profiling of the wild type under 45°C and 70°C after 30 min from transient state.**

The cytosolic and membrane protein were extracted from wild type after transfer to 45°C and 70°C for 30 min, respectively. Each protein sample was separated onto the pH 4 to 7, and 12.5% SDS-PAGE gel (13 cm x 15 cm x 1 mm) and followed CBB G-250 staining with aluminium sulfate. **A**, The cytosolic proteins under 70°C. **B**, The cytosolic proteins under 45°C. **C**, The membrane proteins under 70°C. **D**, The membrane proteins under 45°C.

**Table 3-8. The up-regulated cytosolic proteins in the wild type under cold stress condition (45°C for 30 min).**

orf I.D	Annotated information	Mowse	p/	Mw.	Intensity coverage	Sequence coverage
TTHA0001	DNA polymerase III beta subunit		4.9	40.5	20.2	25.9
TTHA0008	phage shock protein A	112	5.3	25.5	51.0	34.1
TTHA0090	NADH-quinone oxidoreductase chain 3	60	5.6	57.1	24.4	15.2
TTHA0098	arginyl-tRNA synthetase	246	6.6	57.5	55.2	53.5
TTHA0108	transketolase	183	6.1	56.7	47.0	38.5
TTHA0120	GTP-binding protein Era	89	5.9	33.8	57.3	33.6
TTHA0124	branched-chain amino acid aminotransferase(IIvE)	76	5.9	34	41.2	24.4
TTHA0162	30S ribosomal protein S1	50	6.3	57.4	23.9	16.6
TTHA0188	nucleoside diphosphate kinase	59	7.9	15.3	29.3	40.1
TTHA0209	50S ribosomal protein L10	64	?	18.6	66.1	20.2
TTHA0210	50S ribosomal protein L12	100	4.9	13.1	34.7	61.6
TTHA0251	translation elongation factor EF-Tu.B	63	5.3	44.8	14.5	32.5
TTHA0256	leucine aminopeptidase	102	8.7	54.6	37.9	36.1
TTHA0271	60 kDa chaperonin (Protein Cpn60) (GroELprotein)	181	5.1	54.7	71.9	38.1
TTHA0288	2-oxoglutarate dehydrogenase E2 component(dihydrolipoamide succinylt		5.7	44.5	35.8	42.1
TTHA0328	probable isochorismatase	49	5.4	22	72.4	31.6
TTHA0365	type IV pilus assembly protein pilus retractionprotein PilT	71	7.8	39.9	25.6	31.9
TTHA0416	malonyl CoA-[acyl carrier protein] transacylase	205	5.9	33.4	78.2	39.7
TTHA0557	superoxide dismutase [Mn]	107	6.4	23.2	48.6	45.6
TTHA0573	glutamyl-tRNA(Gln) amidotransferase subunit A	172	5.4	50.2	64.9	42.9
TTHA0699	translation initiation factor IF-2	145	5.3	56.4	52.4	45.7
TTHA0701	N utilization substance protein A (NusA)	248	5.7	43.9	50.0	62.8
TTHA1111	alternative ATP-dependent protease La (Lonprotease)	65	5.2	58.4	32.6	20.9
TTHA1276	V-type ATP synthase subunit E	262	5.3	20.6	77.2	63.8
TTHA1343	citrate synthase		6.2	42.3	38.8	41.6
TTHA1482	bacterioferritin	47	4.7	16.2	49.8	36.8
TTHA1577	putative NAD-dependent glutamate dehydrogenase		4.9	44.7	66.5	25.1
TTHA1578	1-pyrroline-5-carboxylate dehydrogenase	97	5.4	56.5	45.0	28.3
TTHA1750	putative mannose-1-phosphate guanylyltransferase (GDP)/mannose-6-	94	5.2	37.4	34.5	30.9
TTHA1769	conserved hypothetical protein	53	6.21	13.1	31.4	51.2
TTHA1779	metal dependent phosphohydrolase (HD domainprotein)	73	5.7	17.8	33.9	38.4
TTHB057	cobalamin biosynthesis protein CbiG	124	6.5	38.7	71.3	46.8
TTHB152	conserved hypothetical protein	186	6.8	51.1	72.2	36.0
TTHB189	conserved hypothetical protein	60	9.6	19.4	14.9	52.7

**Table 3-9. The down-regulated cytosolic proteins in the wild type under cold stress condition (45°C for 30 min).**

orf I.D	Annotated information	Mowse	pI	Mw.	Intensity coverage	Sequence coverage
TTHA0027	probable potassium channel beta subunit(oxidoreductase)	120	5.4	35.9	54.9	42.4
TTHA0122	manganese-containing pseudocatalase	132	5.3	33.3	59.2	40.1
TTHA0407	3-methyl-2-oxobutanoatehydroxymethyltransferase	126	6	28.2	83.4	42.5
TTHA0465	thioredoxin reductase	144	6.5	36.2	53.0	46.6
TTHA0481	oligo-16-glucosidase	119	5.6	59.5	49.5	30.9
TTHA0506	malate synthase	139	6.1	58.7	79.3	23.0
TTHA0562	purine nucleoside phosphorylase	69	5.9	30.3	44.2	33.5
TTHA0614	trigger factor		4.9	46.3	70.4	52.2
TTHA0654	ATP-binding protein Mrp/Nbp35 family	72	5.9	37.2	36.0	24.9
TTHA1131	probable gliding protein (MglB)	77	4.8	17.8	32.3	40.5
TTHA1228	3-isopropylmalate dehydratase large subunit	134	6.4	51.8	62.4	30.1
TTHA1229	3-isopropylmalate dehydratase small subunit	168	4.7	22.6	89.2	54.7
TTHA1248	acetyl-coenzyme A synthetase	83	6	58.4	18.0	34.8
TTHA1373	conserved hypothetical protein	68	6.7	15.7	24.7	40.4
TTHA1427	UvrD protein	51	6.8	57.4	14.8	21.3
TTHA1435	purine nucleoside phosphorylase	138	6.1	25.4	77.4	58.3
TTHA1480	small heat shock protein HSP20 family	69	5.5	15.8	39.8	32.8
TTHA1487	ATP-dependent Clp protease ATP-binding subunitClpB	107	5.5	57.5	40.9	37.1
TTHA1545	hypothetical protein	65	5	19.3	31.3	31.8
TTHA1695	elongation factor G (EF-G)	78	5.2	57.2	43.6	22.3
TTHA1775	pantoate--beta-alanine ligase	49	6.3	30.7	10.4	30.1
TTHA1778	LytR/CspA/Psr family protein	55	6.8	39.7	21.1	19.5
TTHA1809	proline iminopeptidase-related protein	108	4.5	31.5	89.1	24.1
TTHB088	Zn-dependent hydrolase	51	6	30.3	17.3	36.1

**Table 3-10. The up-regulated membrane proteins in the wild type under cold stress condition (45°C for 30 min)**

orf I.D	Annotated information	Mowse	pI	Mw.	Intensity coverage	Sequence coverage
TTHA0075	ribonucleoside-diphosphate reductase	95	6	57.2	26.3	29.5
TTHA0098	arginyl-tRNA synthetase	147	6.6	57.5	81.2	22.5
TTHA0109	ATP-dependent RNA helicase	144	9.6	56.2	76.9	28.5
TTHA0120	GTP-binding protein Era	119	5.9	33.8	79.5	30.5
TTHA0122	manganese-containing pseudocatalase	121	5.3	33.3	59.2	41.4
TTHA0162	30S ribosomal protein S1	59	6.3	57.4	12	35.9
TTHA0229	2-oxoisovalerate dehydrogenase E1 componentalpha subunit	141	5.3	41.1	81.1	22.9
TTHA0232	pyruvate dehydrogenase complex dihydrolipoamideacetyltransferase E2	213	5.6	50.1	65.7	51.7
TTHA0251	translation elongation factor EF-Tu.B	205	5.3	44.8	63.8	51.7
TTHA0271	60 kDa chaperonin (Protein Cpn60) (GroELprotein)	233	5.1	54.7	61	50.2
TTHA0272	10 kDa chaperonin (Protein Cpn10) (groESprotein)	129	5.1	11	37.4	53.5
TTHA0287	2-oxoglutarate dehydrogenase E3 component(dihydrolipoamide dehydr	198	6.8	49	56.9	36.9
TTHA0359	cold shock protein	49	7.6	7.8	31.4	70.6
TTHA0364	type IV pilus assembly protein PilF	123	4.9	56.6	45.5	32.2
TTHA0525	glycine dehydrogenase (decarboxylating) subunit I	59	5.3	47	29.2	16
TTHA0538	succinyl-CoA synthetase beta chain					
TTHA0553	50S ribosomal protein L20	80	12.1	13.7	24.1	40.7
TTHA0614	trigger factor	183	4.9	46.3	86.5	47.8
TTHA0701	N utilization substance protein A (NusA)	209	5.7	43.9	71.9	39
TTHA0770	ATP-dependent protease La (Lon protease)	57	6.1	58.5	37.8	20.7
TTHA0861	30S ribosomal protein S2	154	5.3	29.3	77.1	41.8
TTHA1088	cell division protein FtsA	92	5.7	43.7	38.2	26.5
TTHA1111	alternative ATP-dependent protease La (Lonprotease)	48	5.2	58.4	35.3	11.5
TTHA1123	acetyl-CoA carboxylase biotin carboxylasesubunit	277	6.1	49.3	79.7	58.8
TTHA1148	propionyl-CoA carboxylase alpha subunit	112	5.8	56.3	56.8	25.6
TTHA1210	2-isopropylmalate synthase (LeuA)	56	5.7	56.5	36.7	16
TTHA1211	probable ketol-acid reductoisomerase (IlvC)	114	6.2	37.1	91.1	24.6
TTHA1251	preprotein translocase SecA subunit	135	6.3	58.3	22.3	31.4
TTHA1294	ribosomal subunit interface protein	136	6.4	21.6	49.3	58.1
TTHA1329	glutamine synthetase	53	6.2	50.5	43.6	16.6
TTHA1459	ABC transporter ATP-binding protein	219	6.5	33.9	75.1	68.4
TTHA1483	conserved putative protein	123	5.9	25.7	61.9	56.6
TTHA1487	ATP-dependent Clp protease ATP-binding subunitClpB	64	5.5	57.5	59.2	18.9
TTHA1642	S-adenosylmethionine synthetase		5.5	43.2	63.4	55.7
TTHA1664	DNA-directed RNA polymerase alpha chain	74	4.8	35	27.2	22.2
TTHA1665	30S ribosomal protein S4	110	11.3	24.3	71	27.8
TTHA1689	50S ribosomal protein L2	201	11.7	30.4	65	58
TTHA1696	30S ribosomal protein S7	84	11	18	41.5	32.7
TTHA1783	50S ribosomal protein L21	89	10.5	11	57.4	31.7
TTHA1813	DNA-directed RNA polymerase beta chain (RpoB)	147	6.3	57.8	39.2	41.6
TTHA1818	RecA protein	189	5.5	35.4	72.4	58.5
TTHA1840	SufD protein (membrane protein)	205	61	47.9	46.3	43.6
TTHB190	conserved hypothetical protein	122	5.6	40.8	57.9	30.2

**Table 3-11. The down-regulated membrane proteins in the wild type under cold stress condition (45°C for 30 min).**

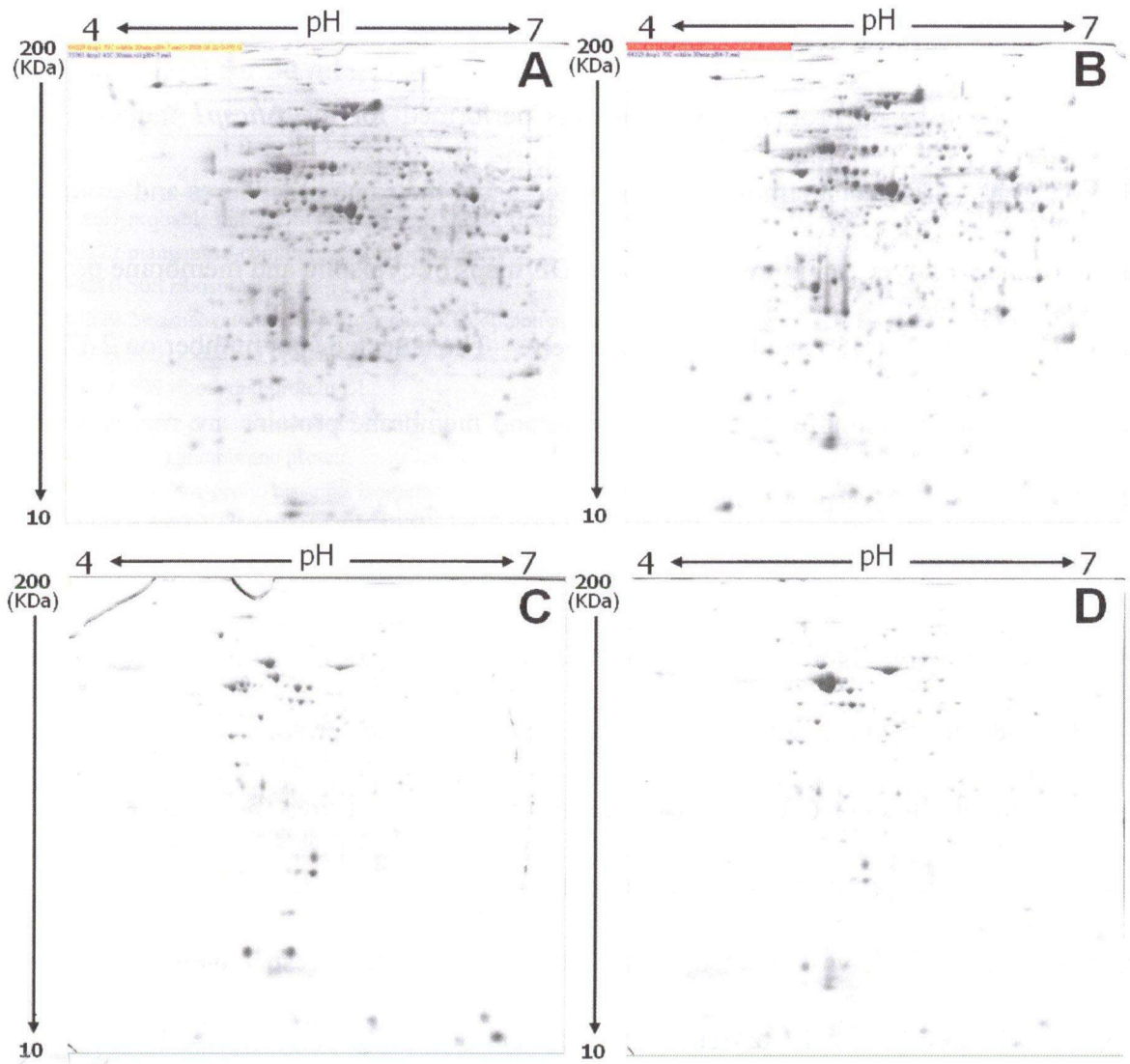
orf I.D	Annotated information	Mowse	<i>pI</i>	Mw.	Intensity coverage	Sequence coverage
TTHA0027	probable potassium channel beta subunit(oxidoreductase)	246	5.4	35.9	56.7	76.9
TTHA0122	manganese-containing pseudocatalase	147	5.3	33.3	62.5	42.1
TTHA0210	50S ribosomal protein L12	100	4.9	13.1	31.4	66.4
TTHA0229	2-oxoisovalerate dehydrogenase E1 componentalpha subunit	131	5.3	41.1	71.2	23.2
TTHA0245	30S ribosomal protein S6 (TS9)	89	7.2	12	52.9	31.7
TTHA0246	50S ribosomal protein L1	179	10	24.8	65.4	58.5
TTHA0286	serine protease subtilase family					
TTHA0561	outer membrane protein	294	4.9	56.2	60.2	55.9
TTHA0602	peptidyl-prolyl cis-trans isomerase	204	5.4	37.4	53.3	58.2
TTHA1272	V-type ATP synthase subunit B	157	5.1	53.1	50.7	38.1
TTHA1273	V-type ATP synthase subunit A	340	5	56.1	73.1	64.8
TTHA1276	V-type ATP synthase subunit E	221	5.3	20.6	60.4	71.8
TTHA1279	V-type ATP synthase subunit (VAPC-THERM)	69	7.3	13.1	40.7	31.7
TTHA1480	small heat shock protein HSP20 family	95	5.5	15.8	68	30
TTHA1570	deoxyhypusine synthase	127	5.7	38.3	68.9	27.5
TTHA1689	50S ribosomal protein L2	72	11.7	30.4	26	32.6
TTHA1695	elongation factor G (EF-G)	86	5.2	57.2	29.3	30.3
TTHA1773	fructose-16-bisphosphate aldolase	59	6	33	48.9	20.3
TTHB182	conserved hypothetical protein	99	5.2	56.2	40.7	28.3

### 3-3 Cold stress response of *Δttcsp1* under 45°C

Next, 2-DE-based proteome analysis was performed for the *Δttcsp1* mutant. The 2-DE map of *Δttcsp1* mutant strain was constructed using the same procedures and compared with that of the wild type. Figure 3-3 shows 2-DE maps of cytosolic and membrane proteins of *Δttcsp1* mutant under 45°C and 70°C, respectively. The detected spot number on 2-DE gel of each group and matching of spots in cytosolic and membrane proteins are represented in Tables 3-1, 3-2 and 3-3, respectively.

#### 3.3.1 Overall trend of proteome change in *Δttcsp1* mutant

The identification results of up- and down-regulated proteins in cytosolic and membrane protein fractions under 45°C are summarized in Tables 3-12 to 3-15. The proteome change in *Δttcsp1* mutant under cold stress condition showed that 21 and 37 proteins were up- and down-regulated in cytosolic protein fraction, respectively (Tables 3-12 and 3-13), whereas 41 and 17 proteins were up- and down-regulated in membrane protein fraction, respectively (Tables 3-14 and 3-15). As a whole, the trend of the proteome change in *Δttcsp1* mutant as cold stress response seems similar to that of the wild type. It should be noted, however, that the the number of up-regulated proteins in cytosolic proteome of *Δttcsp1* mutant was smaller than that of down-regulated ones.



**Figure 3-3. 2-DE proteome profiling of  $\Delta ttcsp1$  mutant under 45°C and 70°C after 30 min from transient state.**

The cytosolic and membrane proteins were extracted from  $\Delta ttcsp1$  mutant after transfer to 45°C and 70°C for 30 min, respectively. Each protein sample was separated onto the pH 4 to 7, and 12.5% SDS-PAGE gel (13 cm x 15 cm x 1 mm) and followed CBB G-250 staining with aluminium sulfate. **A**, The cytosolic proteins under 70°C. **B**, The cytosolic proteins under 45°C. **C**, The membrane proteins of under 70°C. **D**, The membrane proteins under 45°C.



3.3.2 *ttCsp1* also involves the expression of 30S ribosomal protein S1 under cold stress condition.

Significant fraction of the up-regulated proteins in the wild type under cold shock condition was down-regulated in the *Δttcsp1* mutant under 45°C. These includes proteins involved in mRNA stabilizing and translational process (TTHA0162, 30S ribosomal protein S1; TTHA0739, L-serine dehydratase beta subunit; TTHA1586, DNA gyrase B; TTHA0115, prolyl-tRNA synthetase; TTHA0161, leucyl-tRNA synthetase; TTHA1875, threonyl-tRNA synthetase; and TTHA1958, phenylalanyl-tRNA synthetase alpha chain) and amino acid metabolism (TTHA1605, probable acylamino-acid-releasing enzyme; and TTHA1755, acetylmethionine/acetyl-lysine aminotransferase) (Table 3-13). These results demonstrate that *ttCsp1* is involved in the translational process, especially synthesis of aminoacyl-tRNAs, under cold stress condition. Especially, the 30S ribosomal protein S1 was strongly up-regulated in the wild type strains, but down-regulated in *Δttcsp1* mutant under cold stress condition. These results imply that the *ttCsp1* is involved in the expression of 30S ribosomal protein S1 under cold stress condition.

3.3.3 Regulation of the energy production and the fatty acid biosynthesis pathway were not influenced by deletion of *ttcsp1*.

Likewise the wild type, *Δttcsp1* mutant showed down-regulation of several proteins involved in the energy production under cold stress condition. The ATP synthase subunits, TTHA1272, TTHA1273, and TTHA1276, were strongly down-regulated in *Δttcsp1* mutant under 45°C (Table 3-15). The core enzymes related to fatty acid biosynthesis pathway,

including TTHA0229, TTHA0232, TTHA0287, and TTHA0288, were up-regulated in *Δtcspl* mutant (Table 3-14) as in the wild type. These results indicate that regulation of the energy production and the fatty acid biosynthesis pathway were not influenced by deletion of *tcspl* under the cold condition.

**Table 3-12. The up-regulated cytosolic proteins in *Δtcspl* mutant under cold stress condition (45°C for 30 min).**

orf I.D	Annotated information	Mowse	pI	Mw.	Intensity coverage	Sequence coverage
TTHA0214	probable kinase	147	4.9	54.8	42.8	40.6
TTHA0256	leucine aminopeptidase	133	8.7	54.6	61.4	36.7
TTHA0321	polypeptide deformylase	71	5.0	22.1	21.7	35.4
TTHA0537	succinyl-CoA synthetase alpha chain	83	5.5	29.8	80.6	29.9
TTHA0812	phosphoribosylamine--glycine ligase	57	5.3	44.4	14.7	25.9
TTHA1028	thiosulfate sulfurtransferase	72	5.2	32.9	36.1	27.0
TTHA1111	alternative ATP-dependent protease La (Lonprotease)	84	5.2	58.4	39.5	24.4
TTHA1276	V-type ATP synthase subunit E	110	5.3	20.6	35.7	43.1
TTHA1434	3-hydroxybutyryl-CoA dehydratase	79	4.6	29.2	87.3	25.8
TTHA1473	conserved hypothetical protein	101	4.9	16.8	71.3	50.0
TTHA1479	conserved hypothetical protein	80	5.0	16.8	43.3	37.2
TTHA1482	bacterioferritin	104	4.7	16.2	48.6	60.4
TTHA1527	NADPH-quinone reductase	122	8.8	33.8	74.8	44.3
TTHA1615	conserved hypothetical protein	66	5.1	15.4	14.8	57.7
TTHA1624	conserved hypothetical protein	155	5.0	27.4	75.6	60.3
TTHA1714	conserved hypothetical protein	55	5.7	28.9	23.6	34.1
TTHA1914	homocitrate synthase	177	5.8	42.1	48.6	50.3
TTHB023	transcriptional regulator TetR family	51				
TTHB057	cobalamin biosynthesis protein CbiG	159	6.5	38.7	68.8	48.5
TTHB125	chromosome partitioning ATPase ParA family	183	6.6	35.9	84.1	34.2
TTHB179	conserved hypothetical protein	114	9.8	23.9	46.2	48.6

**Table 3-13. The down-regulated cytosolic proteins in *Δttcsp1* mutant under cold stress condition (45°C for 30 min).**

orf ID	Annotated information	Mowse	<i>pI</i>	Mw.	Intensity coverage	Sequence coverage
TTHA0001	DNA polymerase III beta subunit	72	4.9	40.5	22.4	32.3
TTHA0115	prolyl-tRNA synthetase	139	5.9	54.5	52.8	43.4
TTHA0122	manganese-containing pseudocatalase	130	5.3	33.3	61.1	38.7
TTHA0136	LAO/AO transport system kinase	180	6.4	33.5	54.2	53.0
TTHA0161	leucyl-tRNA synthetase	110	6.0	59.2	62.2	32.6
TTHA0162	30S ribosomal protein S1	216	6.3	57.4	54.7	50.0
TTHA0232	pyruvate dehydrogenase complex dihydrolipoamideacetyltransferase E2 compo	109	5.6	50.1	36.3	32.2
TTHA0256	leucine aminopeptidase	115	8.7	54.6	48.0	37.3
TTHA0271	60 kDa chaperonin (Protein Cpn60) (GroELprotein)	89	5.1	54.7	63.3	28.1
TTHA0380	aldehyde:ferredoxin oxidoreductase	54	6.7	54.7	41.4	21.5
TTHA0512	conserved hypothetical protein	93	6.3	39.2	46.4	29.6
TTHA0520	NAD-dependent malic enzyme (malatedehydrogenase)	106	6.2	57.4	20.7	41.0
TTHA0558	fumarate hydratase class II (EC 4.2.1.2)	169	6.3	50.9	86.9	33.9
TTHA0591	UDP-glucose 4-epimerase	139	6.2	33.8	62.3	46.9
TTHA0599	protoporphyrinogen oxidase (HemG)	63	10.5	49.2	13.9	34.6
TTHA0634	magnesium chelatase related protein	245	5.7	51.4	77.4	57.1
TTHA0704	pyridoxine biosynthesis protein	61	5.4	32.4	17.3	20.2
TTHA0739	L-serine dehydratase beta subunit	48	6.4	23.6	54.6	26.8
TTHA0968	phenylacetic acid degradation protein PaaZ	102	6.4	55.2	56.8	21.3
TTHA1097	DNA ligase [NAD <sup>+</sup> ]	107	7.8	58.9	30.5	36.1
TTHA1124	acetyl-CoA carboxylase biotin carboxyl carrierprotein	57	4.5	17.7	47.5	38.2
TTHA1429	metallo-beta-lactamase family protein	113	6.2	35.4	68.3	41.0
TTHA1527	NADPH-quinone reductase	137	8.8	33.8	75.8	55.7
TTHA1569	hypothetical protein	203	5.3	57.5	44.9	43.0
TTHA1576	NAD-dependent glutamate dehydrogenase	138	6.8	43.4	9.8	24.1
TTHA1586	DNA gyrase subunit B	89	6.1	55.8	31.4	31.1
TTHA1605	probable acylamino-acid-releasing enzyme	94	5.4	56.9	43.3	20.5
TTHA1637	ribose-phosphate pyrophosphokinase	76	5.9	33.5	86.3	32.6
TTHA1659	tetratricopeptide repeat family protein	186	6.1	48.9	49.4	45.0
TTHA1735	iron-sulfur cluster biosynthesis protein IscS(cysteine desulfurase)	198	6.3	44.7	58.6	48.3
TTHA1755	acetylmornithine/acetyl-lysine aminotransferase	137	6.8	43.4	48.5	58.5
TTHA1774	pili retraction protein PilT	62	6.2	41.1	42.2	17.1
TTHA1813	DNA-directed RNA polymerase beta chain (RpoB)	146				
TTHA1875	threonyl-tRNA synthetase	138	5.6	58.8	56.8	30.1
TTHA1958	phenylalanyl-tRNA synthetase alpha chain	219	5.7	39.2	92.1	46.3
TTHB125	chromosome partitioning ATPase ParA family	89	6.6	35.9	28.6	29.5
TTHB163	conserved hypothetical protein	210	5.6	30.7	50.6	59.8

**Table 3-14. The up-regulated membrane proteins in *Δtscp1* mutant under cold stress condition (45°C for 30 min).**

orf I.D	Annotated information	Mowse	p/	Mw.	Intensity coverage	Sequence coverage
TTHA0090	NADH-quinone oxidoreductase chain 3	74	5.6	57.1	32.3	13.9
TTHA0098	arginyl-tRNA synthetase	147	6.6	57.5		
TTHA0120	GTP-binding protein Era	119	5.9	33.8		
TTHA0122	manganese-containing pseudocatalase	121	5.3	33.3		
TTHA0158	alpha-dextran endo-16-alpha-glucosidase((amylo)pullulanase)	109	6.0	58.9	28.6	21.3
TTHA0206	nicotinamide nucleotide transhydrogenase alphasubunit 1	103	6.9	39.9	51.6	17.1
TTHA0229	2-oxoisovalerate dehydrogenase E1 componentalpha subunit	141	5.3	41.1		
TTHA0230	2-oxoisovalerate dehydrogenase E1 componentbeta subunit		5.8	35.1	29.4	24.7
TTHA0232	pyruvate dehydrogenase complex dihydrolipoamideacetyltransferase E2 compor	213	5.6	50.1		
TTHA0251	translation elongation factor EF-Tu.B					
TTHA0271	60 kDa chaperonin (Protein Cpn60) (GroELprotein)	233	5.1	54.7		
TTHA0272	10 kDa chaperonin (Protein Cpn10) (groESprotein)		5.1	11.0	8.4	68.3
TTHA0287	2-oxoglutarate dehydrogenase E3 component(dihydrolipoamide dehydroge	198	6.8	49.0		
TTHA0288	2-oxoglutarate dehydrogenase E2 component(dihydrolipoamide succinylt	135	5.7	44.5		
TTHA0304	enoyl-[acyl carrier protein] reductase		5.8	28.1	9.4	25.7
TTHA0364	type IV pilus assembly protein PilF	123	4.9	56.6		
TTHA0442	probable DNA/RNA-binding protein (Jag-relatedprotein)		6.1	21.0	8.7	25.9
TTHA0525	glycine dehydrogenase (decarboxylating) subunit1	59	5.3	47.0		
TTHA0614	trigger factor	183	4.9	46.3		
TTHA0701	N utilization substance protein A (NusA)	209	5.7	43.9		
TTHA0702	conserved hypothetical protein	136	4.9	17.8	82.8	58.0
TTHA0770	ATP-dependent protease La (Lon protease)	57	6.1	58.5		
TTHA0861	30S ribosomal protein S2	154	5.3	29.3		
TTHA1111	alternative ATP-dependent protease La (Lonprotease)	48	5.2	58.4		
TTHA1123	acetyl-CoA carboxylase biotin carboxylasesubunit	277	6.1	49.3		
TTHA1210	2-isopropylmalate synthase (LeuA)		5.7	56.5	38.9	27.0
TTHA1211	probable ketol-acid reductoisomerase (IlvC)	114	6.2	37.1		
TTHA1243	septum site-determining protein MinD	52	5.3	28.9	28.4	19.1
TTHA1251	preprotein translocase SecA subunit	135	6.3	58.3		
TTHA1294	ribosomal subunit interface protein	134	6.4	21.6	54.6	43.5
TTHA1459	ABC transporter ATP-binding protein	219	6.5	33.9		
TTHA1483	conserved putative protein	123	5.9	25.7		
TTHA1484	small heat shock protein HSP20 family	91	5.8	15.7	64.5	46.7
TTHA1487	ATP-dependent Clp protease ATP-binding subunitClpB	64	5.5	57.5		
TTHA1642	S-adenosylmethionine synthetase		5.5	43.2		
TTHA1665	30S ribosomal protein S4	134	11.3	24.3	74.3	48.3
TTHA1689	50S ribosomal protein L2	201	11.7	30.4		
TTHA1696	30S ribosomal protein S7	84	11.0	18.0		
TTHA1813	DNA-directed RNA polymerase beta chain (RpoB)	147	6.3	57.8		
TTHA1818	RecA protein	189	5.5	35.4		
TTHA1840	SufD protein (membrane protein)	205	61.0	47.9		

**Table 3-15. The down-regulated membrane proteins in *Δttcsp1* mutant under cold stress condition (45°C for 30 min).**

orf I.D	Annotated information	Mowse	pI	Mw.	Intensity coverage	Sequence coverage
TTHA0122	manganese-containing pseudocatalase	98	5.3	33.3	55.7	27.2
TTHA0210	50S ribosomal protein L12	54	4.9	13.1	23.0	32.0
TTHA0229	2-oxoisovalerate dehydrogenase E1 component alpha subunit	149	5.3	41.4	67.6	23.2
TTHA0245	30S ribosomal protein S6 (TS9)	142	7.2	12.0	35.7	62.4
TTHA0251	translation elongation factor EF-Tu.B	116	5.3	44.8	54.0	26.6
TTHA0271	60 kDa chaperonin (Protein Cpn60) (GroELprotein)	260	5.1	54.7	59.0	50.0
TTHA0561	outer membrane protein	209	4.9	56.2	72.0	35.5
TTHA0602	peptidyl-prolyl cis-trans isomerase	204	5.4	37.4	53.3	58.2
TTHA0906	phosphoglycerate kinase	151	5.5	41.8	43.0	40.0
TTHA1210	2-isopropylmalate synthase (LeuA)	107	5.7	56.5	61.4	21.1
TTHA1272	V-type ATP synthase subunit B	64	5.1	53.1	68.4	20.7
TTHA1273	V-type ATP synthase subunit A	340	5.0	56.1	73.1	64.8
TTHA1276	V-type ATP synthase subunit E	221	5.3	20.6	60.4	71.8
TTHA1484	small heat shock protein HSP20 family	71	5.8	15.7	52.6	34.3
TTHA1570	deoxyhypusine synthase	101	5.7	38.3	83.3	16.2
TTHA1694	elongation factor Tu (EF-Tu)	133	5.3	44.8	65.6	31.0
TTHA1839	SufB protein (membrane protein)	106	5.2	53.1	39.1	26.7

### 3-4 Difference of proteome results between wild type and the *Δttcsp1* mutant under cold stress condition.

I focused on proteins that showed different expression pattern between the wild type and *Δttcsp1* mutant strains under cold stress condition (at 45°C). The gel-to-gel matching of spots (wild type vs. the *Δttcsp1* mutant) showed 74.5% (G-V vs. G-VI) and 62.0% (G-IX vs. G-X) for cytosolic and membrane protein fractions, respectively (Tables 3-2 and 3-3). Then I identified up- and down-regulated proteins (with more than 1.5-fold change,  $p < 0.05$ ) in the *Δttcsp1* mutant grown at 45°C against the wild type grown at the same temperature. 20 up-regulated proteins (Table 3-16) and 28 down-regulated proteins (Table 3-17) were identified in cytosolic protein fraction, and 7 up-regulated (Table 3-18) and 11 down-regulated proteins (Table 3-19) were identified in membrane protein fraction.

Under cold stress condition, EF-Tu (TTHA0251 and TTHA1694), NusA (TTHA0701), and *ttCsp2* (TTHA0359) were identified as cold stress response proteins in the wild type strain (Tables 3-8 and 3-10), but were down-regulated in the  $\Delta$ *ttcsp1* mutant (Tables 3-17 and 3-19). These proteins were also down-regulated in the  $\Delta$ *ttcsp1* mutant even at 70°C. These results indicate that the *ttcsp1* deletion resulted in the down-regulation of cold shock responsible proteins under both optimal and cold stress conditions (70°C and 45°C).

I also identified 13 candidates for proteins of which expression may be affected by *ttCsp1*. By comparative proteome analysis, I extracted the proteins which were commonly found both in up-regulated protein list of the wild type at 45°C (G-III vs. G-V (Table 3-8) and G-VII vs. G-IX (Table 3-10)) and down-regulated protein list of the  $\Delta$ *ttcsp1* mutant at optimal growth temperature (G-III vs. G-IV (Table 3-5) and G-VII vs. G-VIII (Table 3-7)). These are TTHA0090 (NADH-quinone oxidoreductase chain 3), TTHA0098 (arginyl-tRNA synthetase), TTHA0229 (2-oxoisovalerate dehydrogenase, E1 component  $\alpha$  subunit), TTHA0271 (60 kDa chaperonin), TTHA 0557 (superoxide dismutase), TTHA 0614 (trigger factor), TTHA0699 (IF2), TTHA1123 (acetyl-CoA carboxylase biotin carboxylase subunit), TTHA1483 (conserved hypothetical protein), TTHA1487 (ClpB), TTHA1642 (S-adenosylmethionine synthetase), TTHA1818 (RecA), and TTHB152 (CRISPR-associated protein).

**Table 3-16. The up-regulated cytosolic proteins in *Δttcsp1* mutant under cold stress condition (45°C for 30 min).**

orf I.D	Annotated information	Mowse	pI	Mw.	Intensity coverage	Sequence coverage
TTHA0141	hypothetical protein	148	4.9	16.7	57.5	72.8
TTHA0271	60 kDa chaperonin (Protein Cpn60) (GroEL)protein		5.1	54.7	21.4	11.3
TTHA0614	trigger factor	124	4.9	46.3	38.8	31.9
TTHA0699	translation initiation factor IF		5.3	56.4	51.3	24.0
TTHA0860	elongation factor Ts (EF-Ts)	80	6.2	22.4	44.2	37.8
TTHA0924	conserved hypothetical protein	198	5.3	36.0	74.5	46.9
TTHA0970	phenylacetic acid degradation protein PaaC	150	4.7	28.7	61.5	39.5
TTHA1169	valyl-tRNA synthetase (valine--tRNA ligase)(ValRS)	84	6.0	59.1	44.1	18.0
TTHA1243	septum site-determining protein MinD	248	5.3	28.9	73.7	58.4
TTHA1479	conserved hypothetical protein	107	5.0	16.8	43.7	45.3
TTHA1498	elongation factor G (EF-G-2)	134	5.1	56.7	31.0	28.5
TTHA1519	phosphoribosylformylglycinamide synthase II	137	5.5	55.7	55.7	32.8
TTHA1602	conserved hypothetical protein	67	5.3	11.9	12.5	44.2
TTHA1610	conserved hypothetical protein	110	5.5	8.7	57.4	75.3
TTHA1625	osmotically inducible protein OsmC		5.4	15.3	30.5	44.4
TTHA1670	methionine aminopeptidase		5.4	27.8	61.5	53.3
TTHA1695	elongation factor G (EF-G)		5.2	57.2	42.1	35.7
TTHA1519	phosphoribosylformylglycinamide synthase II					
TTHA1938	acyl-CoA dehydrogenase	181	5.5	41.5	70.4	35.7
TTHA1958	phenylalanyl-tRNA synthetase alpha chain	114	5.7	39.2	75.3	24.0

**Table 3-17. The down-regulated cytosolic proteins in *Δttcsp1* mutant under cold stress condition (45°C for 30 min).**

orf I.D	Annotated information	Mowse	pI	Mw.	Intensity coverage	Sequence coverage
TTHA0090	NADH-quinone oxidoreductase chain 3	165	5.6	57.1	62.6	26.4
TTHA0108	transketolase	101	6.1	56.7	18.1	35.9
TTHA0116	phosphonopyruvate decarboxylase	97	6.1	44.4	39.7	32.8
TTHA0245	30S ribosomal protein S6 (TS9)	60				
TTHA0278	ATP-dependent phosphoenolpyruvate carboxykinase	203	6.4	57.6	71.5	41.0
TTHA0359	cold shock protein, Csp2	69	7.6	7.8	40.4	82.4
TTHA0512	conserved hypothetical protein	123	6.3	39.2	62.6	31.3
TTHA0520	NAD-dependent malic enzyme (malatedehydrogenase)	93	6.2	57.4	23.0	21.9
TTHA0525	glycine dehydrogenase (decarboxylating) subunit1	148	5.3	47.1	83.2	24.4
TTHA0614	trigger factor	206	4.9	46.3	58.6	50.0
TTHA0630	heat shock protein HslU	220	5.6	46.5	59.7	51.2
TTHA0634	magnesium chelatase related protein	153	5.7	51.4	71.0	40.1
TTHA0704	pyridoxine biosynthesis protein	158	5.4	32.4	43.9	32.7
TTHA0989	conserved hypothetical protein	137	5.6	40.3	32.8	40.2
TTHA1124	acetyl-CoA carboxylase biotin carboxyl carrierprotein	55	4.5	17.7	25.8	29.7
TTHA1197	N-acetyl-gamma-glutamyl-phosphate reductase	115	6.0	37.6	24.2	30.7
TTHA1274	V-type ATP synthase subunit F	46	4.5	11.3	6.0	67.3
TTHA1447	alanine dehydrogenase	85	5.9	36.4	45.1	28.7
TTHA1577	putative NAD-dependent glutamate dehydrogenase	142	4.9	44.7	74.0	27.2
TTHA1589	50S ribosomal protein L25 (TL5)	134	5.1	23.2	55.0	35.0
TTHA1637	ribose-phosphate pyrophosphokinase	102	5.9	33.5	67.9	43.9
TTHA1694	elongation factor Tu (EF-Tu)	231	5.3	44.8	60.7	56.2
TTHA1779	metal dependent phosphohydrolase (HD domainprotein)	210	5.7	17.8	60.8	64.2
TTHA1839	SufB protein (membrane protein)	131	5.2	53.1	38.1	32.7
TTHA1875	threonyl-tRNA synthetase	115	5.6	58.8	34.6	33.0
TTHA1958	phenylalanyl-tRNA synthetase alpha chain	58	5.7	39.2	13.7	20.6
TTHB057	cobalamin biosynthesis protein CbiG	168	6.5	38.7	49.4	51.3
TTHB179	conserved hypothetical protein	59				

**Table 3-18. The up-regulated membrane proteins in *Δttcsp1* mutant under cold stress condition (45°C for 30 min).**

orf I.D	Annotated information	Mowse	pI	Mw.	Intensity coverage	Sequence coverage
TTHA0233	pyruvate dehydrogenase complex dihydrolipoamidedehydrogenase E3 component	94	6.4	49.1	49.8	17.7
TTHA0288	2-oxoglutarate dehydrogenase E2 component(dihydrolipoamide succinylt	78	5.7	44.5	42.9	22.7
TTHA0561	outer membrane protein	184	4.9	56.2	33.7	38.1
TTHA0602	peptidyl-prolyl cis-trans isomerase	231	5.4	37.4	66.7	65.9
TTHA0770	ATP-dependent protease La (Lon protease)	53	6.1	58.5	43.0	18.8
TTHA0861	30S ribosomal protein S2	126	5.3	29.0	46.1	53.9
TTHA1383	general secretion pathway protein (PilQ)	236	6.5	56.0	72.4	43.9



**Table 3-19. The down-regulated membrane proteins in *Δttcsp1* mutant under cold stress condition (45°C for 30 min).**

orf I.D	Annotated information	Mowse	pI	Mw.	Intensity coverage	Sequence coverage
TTHA0122	manganese-containing pseudocatalase	67	5.3	33.3	59.3	28.3
TTHA0229	2-oxoisovalerate dehydrogenase E1 component alpha subunit	138	5.3	41.1	67.3	21.5
TTHA0251	translation elongation factor EF-Tu.B	124	5.3	44.8	86.7	32.5
TTHA0701	N utilization substance protein A (NusA)	67	5.7	43.0	38.9	31.0
TTHA1210	2-isopropylmalate synthase (LeuA)	228	5.7	56.5	57.4	50.0
TTHA1294	ribosomal subunit interface protein	140	6.4	21.6	72.6	55.9
TTHA1642	S-adenosylmethionine synthetase	257	5.5	43.2	59.4	60.0
TTHA1665	30S ribosomal protein S4	151	11.3	24.0	79.6	53.1
TTHA1689	50S ribosomal protein L2	236	11.7	30.4	86.2	66.3
TTHA1695	elongation factor G (EF-G)	122	5.2	57.2	59.3	28.9
TTHA1813	DNA-directed RNA polymerase beta chain (RpoB)	66	6.3	57.0	33.6	15.8

At least part of the proteins whose expression level was caused by *ttcsp1* deletion is considered to be controlled by the cold shock response pathway involving *ttCsp1*. It should be mentioned, however, that the *Δttcsp1* mutant showed similar growth rate to the wild type strain under both optimal and cold stress conditions (Figure 1-1B). The *Δttcsp1* mutant led to significant down-regulation of *ttCsp2*, the other cold shock-induced protein. Nevertheless, this mutant strain was rapidly adapted to cold stress condition. Such fast adaptation to cold stress condition without *ttCsp1* and *ttCsp2* in *Δttcsp1* mutant raises the possibility that an alternative cold-adaptation mechanism exists in *T. thermophilus* HB8.

As up-regulated proteins under cold stress condition without *ttCsp1* and *ttCsp2* in the *Δttcsp1* mutant (Tables 3-16 and 3-18), elongation factor Ts (EF-Ts, TTHA0860), elongation factor G2 (TTHA1498), osmotically inducible protein OsmC (TTHA1625) and elongation factor G (TTHA1695) in cytosolic protein fraction, and 30S ribosomal protein S2 (TTHA0861), outer membrane protein (TTHA0561), general secretion pathway protein (TTHA1383) in membrane protein fraction were newly identified. Although most of them

belong to proteins involved in translation process, it is uncertain at present whether these proteins are related to alternative cold-adaptation mechanism. In addition, it was also noted that six hypothetical proteins (TTHA0141, TTHA0924, TTHA1479, TTHA1498, TTHA1602 and TTHA1610) were also up-regulated. Also among the up-regulated proteins (45°C vs. 70°C) in the *Δttcsp1* mutant under cold stress condition, seven hypothetical proteins (TTHA1473, TTHA1479, TTHA1482, TTHA1615, TTHA1624, TTHA1714 and TTHB179) were identified. The study to elucidate functional role of these hypothetical proteins will be needed to verify the relationship to stress response mechanism.

#### 4. Comparison of transcriptome and proteome results

I obtained two types of data sets from transcriptome and proteome analyses. Making use of these data, I could obtain more information about the functions of *tCsp1*. Although I have not finished identification of all protein spots on the gels under each condition (some spots remained to be identified, because their amounts were too small to be detected or their pIs were out of range), I could compare the result from proteome with that from microarray.

Here I classify the genes identified by the proteome analysis into three groups (A to C) according to the relationship to the transcriptome data. Group A contains the proteins whose expression changes were detected also in the transcriptome samples under the same condition, and 24 proteins can be classified into Group A (Table 4-1). As shown in Table 4-1, up-regulated protein under certain condition (for example, TTHA1498) were also up-regulated on the (*ttha1498*) transcription level, and down-regulated proteins (for example, TTHA1695) were also down-regulated on the (*ttha1695*) transcription level. Because the expression changes were correlated with the changes of the mRNA, it means the expression patterns of proteins might be directly influenced by the amount of mRNAs in these cases.

In regard to some proteins, the changing ratio calculated from microarray analysis was not identical to the ratio from proteome analysis. It is likely that *tCsp1* helped the degradation of the proteins or decreased the translation efficiency. On the other hand, mRNAs of *tCsp2* (Group A) were increased only 2.7-fold under the cold shock condition in the wild type, its protein spot intensity of the *tCsp2* was increased more than 50-fold. This raises the possibility that *tCsp1* functions not only as a transcriptional regulator but also as a

translational regulator. This hypothesis might be supported by existence of Group B proteins whose expression changes were detected only from proteome analysis.

More than 50% of proteins are classified into Group B, whose expression changes were detected only by proteome analysis. For example, the expression pattern of TTHA0699 (translation initiation factor IF-2) was decreased even though the amounts of mRNAs were not affected by deletion of *ttcsp1* gene. Furthermore, some proteins belonging to Group B were increased at protein level in the  $\Delta ttcsp1$  mutant, even though the mRNA transcripts were not changed. Therefore, it is inferred that *ttCsp1* can both suppress and enhance translation independent of transcription. It was already reported that *ecCspA* and *ecCspE* are involved in mRNA stabilization to increase life time of mRNA through a protection from cellular RNases (Feng, Y. *et al.*, 2001). However, up-regulation of Group B proteins could not be explained only by stabilization of the mRNAs because their mRNA levels were not changed. Some cold-inducible Csps from other organisms are thought to promote translational repression by destabilizing mRNA structures (Phadtare, S. and Inouye, M. 1999). However, this is the first report to indicate that a Csp family protein facilitates translation without altering mRNA levels under optimal growth conditions. The results suggest that *ttCsp1* can suppress aspects of cellular metabolism and alter the properties of protein production machinery. These results also imply that *ttCsp1* can sense minor fluctuations in the growth conditions and prepare for the stress response. There might be a regulation system controlling the initiation or elongation of translation.

Some proteins belonging to Group C made a protein spot at the unexpected position, or made several spots on the same gel. TTHA0271 (GroEL), TTHA0614 (trigger factor) and

some ribosomal proteins are the members of Group C. Such phenomena might reflect the changes of protein maturation states, folding states and modification patterns after translation event. The proteins identified from membrane protein fraction are classified into Group C at higher frequency than cytosolic proteins. I have not been able to identify the reasons of spots shift completely yet, however, with regard to GroEL, phosphorylation and acetylation were detected. Although the functions of these modifications have not been clear, the ratio of modification forms of chaperonin and translational regulators were changed in the absence of *ttCsp1*. Deletion of *ttcsp1* may cause the alteration of the frequency of protein synthesis.

**Table 4-1. Genes classified into Group A.**

Group A contains the genes which showed similar expression changes both in the transcriptome and proteome samples under the same conditions.

Functional category	orf I.D	Annotated information
<i>Δ ttcspI</i> > WT (at 70°C)		
translation	TTHA1498	elongation factor G (EF-G-2)
<i>Δ ttcspI</i> < WT (at 70°C)		
transcription	TTHA0248	transcription antitermination protein NusG
translation	TTHA1695	elongation factor G (EF-G)
Metabolism of Carbohydrate	TTHA1066	probable transaldolase
45°C > 70°C (in WT)		
Csp2	TTHA0359	cold shock protein
RNA helicase	TTHA0109	ATP-dependent RNA helicase
Genetic Information Processing	TTHA0008	phage shock protein A
45°C < 70°C (in WT)		
LytR/CspA/Psr family	TTHA1778	LytR/CspA/Psr family protein
Metabolism of carbohydrate	TTHA0506	malate synthase
Metabolism of Cofactors and Vitamins	TTHA1775	pantoate-β-alanine ligase
Metabolism of energy	TTHA1272	V-type ATP synthase subunit B
Metabolism of energy	TTHA1273	V-type ATP synthase subunit A
Metabolism of energy	TTHA1276	V-type ATP synthase subunit E
45°C > 70°C (in <i>Δ ttcspI</i> )		
transcription	TTHB023	transcriptional regulator, TetR family
translation	TTHA1570	deoxyhypusine synthase
Metabolism of amino acid	TTHA1642	S-adenosylmethionine synthetase
Membrane/ Transporter/ Lipoprotein	TTHA0120	GTP-binding protein Era
Metabolism of amino acid	TTHA1914	homocitrate synthase
hypothetical protein	TTHA0702	hypothetical protein
45°C < 70°C (in <i>Δ ttcspI</i> )		
Chaperones	TTHA1487	ATP-dependent Clp protease, ATP-binding subunit ClpB
Metabolism of amino acid	TTHA0525	glycine dehydrogenase (decarboxylating) subunit 1
Metabolism of Carbohydrate	TTHA0232	pyruvate dehydrogenase complex, dihydroliipoamide acetyltransferase E2 component
Metabolism of Cofactors and Vitamins	TTHA0206	nicotinamide nucleotide transhydrogenase, alpha subunit 1
Genetic Information Processing	TTHA1774	pili retraction protein PilT

## 5. Crystal structure of *ttCsp1*

### 5-1 Overall structure

The crystal structure of *ttCsp1* was determined at 1.65 Å resolution by molecular-replacement method (Table 5-1). The asymmetric unit contains two molecules (A and B). With the exception of loop4, the structure of these structures are almost the same. Therefore, the molecule A was used for the following analyses.

The overall structure of *ttCsp1* is shown in Figure 5-1B, C. *ttCsp1* is composed entirely of an antiparallel five-stranded  $\beta$ -sheet ( $\beta$ 1– $\beta$ 5) with connecting a turn and loops (trun1 and loop2–loop4). The five-stranded antiparallel  $\beta$ -barrel of *ttCsp1* is characteristic of the defined oligonucleotide/oligosaccharide-binding fold (OB-fold, Murzin, A. G., 1993) as shown in Figure 5-1C. Strands  $\beta$ 1 to  $\beta$ 4 of the barrel form the Greek key motif which is common to many known proteins with beta-barrels and almost all proteins with beta-sandwich structures (Zhang, C. *et al.*, 2000).

### 5-2 Structural comparison with cold shock proteins and the Y-box binding protein

Cold shock proteins exist in almost bacteria and the cold shock domain (CSD) is a nucleic acid-binding domain of the eukaryotic gene-regulatory Y-box factors which are involved in transcriptional and translational regulation for messenger RNA (mRNA) and for a wide range of genes containing the Y-box sequence (5'-CTGATTGGCCAA-3') (Ladomery, M., 1997). In addition to the structure of *ttCsp1*, the structures of cold shock proteins from six species of bacteria and a structure of the CSD of human Y-box binding protein 1 (*hsYB1*;

PDB code, 1H95; Kolks, C. P. A. M. *et al.*, 2002) have been determined so far. A sequence alignment of *ttCsp1*, *ttCsp2*, cold shock proteins from six bacteria and CSD of *hsYB1* is shown in Figure 5-1A. The sequence identities of the *ttCsp1* with *ttCsp2*, *bcCsp*, *bsCspB*, *tmCsp*, *ecCspA*, *nmCsp*, *stCspE* and *hsYB1* are 72%, 63%, 59%, 54%, 55%, 55%, 55% and 50%, respectively, showing that the amino acid sequences are conserved among not only bacterial cold shock proteins but also the eukaryotic CSDs.

The three dimensional structures of cold shock proteins have been determined not only by using wild type proteins, mutant proteins and complex with single stranded DNA (ssDNA), and but also by using crystallographic and NMR methods. Therefore, the structural analyses were carried out using the following structures, cold shock protein from *B. caldolyticus* (*bcCsp*), A chain in 1C9O (Mueller, U. *et al.*, 2000); CspB from *Bacillus subtilis* (*bsCspB*), A chain in 1CSP (Schindelin, H., *et al.*, 1993); CspA from *E. coli* (*ecCspA*), A chain in 1MJC (Schindelin, H., *et al.*, 1994); cold shock protein from *Thermotoga martima* (*tmCsp*), A chain in 1G6P (Kremer, W. *et al.*, 2001); cold shock protein from *Neisseria meningitidis* (*nmCsp*), 1–38 residues of A chain and 39–67 of B chain in 3CAM (Kremer, W. *et al.*, 2001); CspE from *Salmonella typhimurium* (*stCspE*), A chain in 3I2Z (Morgan, HP. *et al.*, 2009) and the CSD of *hsYB1*, A chain in 1H95 (Kolks, C. P. A. M. *et al.*, 2002).

To analysis the structural similarity, least-squares fitting of main-chain atoms were carried out using the residues 2–21, 25–51, 62–68 of *ttCsp1* (216 atoms) and the corresponding atoms of six cold shock proteins and CSD of YB1. *nmCsp* forms a dimer by exchange of two  $\beta$ -strands,  $\beta$ 4 and  $\beta$ 5 in the crystal structure, although *nmCsp* behaved as a monomer in solution. Therefore, the residues 2–38 in A chain and residues 39–67 in B



chain of *nmCsp* were used to calculate the r.m.s. deviation. The r.m.s. deviations from the structure of *ttCsp1* are 0.96 in the structure of *bcCsp*, 1.13 of *bsCspB*, 1.06 of *ecCspA*, 1.53 of *nmCsp*, 1.90 of *tmCsp*, 1.27 of *stCspE* and 3.16 of CSD of *hsYB1*, respectively. As shown in Figure 5-1F, the secondary structures of  $\beta$ -barrel are almost same and there are local differences in loop 3 and especially in loop 4. The r.m.s. deviation of CSD of *hsYB1* from *ttCsp1* is higher than those of other cold shock proteins. The five-stranded antiparallel  $\beta$ -barrel consists of the front  $\beta$  sheet containing strands  $\beta 1$ – $\beta 3$  and the back  $\beta$  sheet containing  $\beta 4$  and  $\beta 5$ . As shown in Figure 5-1A, four residues are inserted in loop 3 of CSD of *hsYB1*, forming larger loop 3 than that of *ttCsp1* between the front and back  $\beta$  sheets (Figure 5-1G). Since the r.m.s. deviations from the front  $\beta$  sheet of *ttCsp1* (117 atoms) are 1.04 in the front  $\beta$  sheet of *bcCsp* and 1.39 of CSD of *hsYB1*, the structures of the front  $\beta$  sheet are conserved in not only the cold shock proteins but also CSD of *hsYB1*, suggesting the larger loop3 of CSD of *hsYB1* changes the orientation of the front and back  $\beta$  sheets in the structure of CSD. These structural similarities and differences could emphasize again that the front  $\beta$  sheet containing RNP1 and RNP2 motifs is highly conserved in not only bacterial cold shock protein, but also in eukaryotic CSDs and that RNP1 and RNP2 motifs play an important role to nucleotide binding. These results indicate that the 3D structure of *ttCsp1* is quite similar to those of other Csps and the CSD, suggesting that *ttCsp1* possesses similar molecular properties including the ability to bind DNA.

**Table 5-1. Data collection, refinement and model statistics****Data collection**

Beamline	BL44B2, SPring-8
Wavelength (Å)	1.00
Space group	<i>P</i> 1
Unit-cell parameters	$a = 28.22 \text{ \AA}$ , $b = 29.86 \text{ \AA}$ , $c = 38.26 \text{ \AA}$ $\alpha = 67.37^\circ$ , $\beta = 81.44^\circ$ , $\gamma = 79.49^\circ$
Resolution range <sup>a</sup> (Å)	50.0–1.65 Å (1.71–1.65 Å)
No. of measured reflections	50,732
No. of unique reflections	12,948
Redundancy <sup>a</sup>	3.9 (3.7)
Completeness <sup>a</sup> (%)	95.2% (87.4%)
$R_{\text{merge}}$ <sup>a,b</sup> (%)	3.1% (9.5%)
Average $I/\sigma(I)$ <sup>a</sup>	46.9 (13.9)

**Refinement statistics**

Resolution range <sup>a</sup> (Å)	50.0–1.65 Å (1.75–1.65 Å)
$R_{\text{work}}$ <sup>a</sup>	16.5% (20.0%)
$R_{\text{free}}$ <sup>a</sup>	17.4% (19.6%)

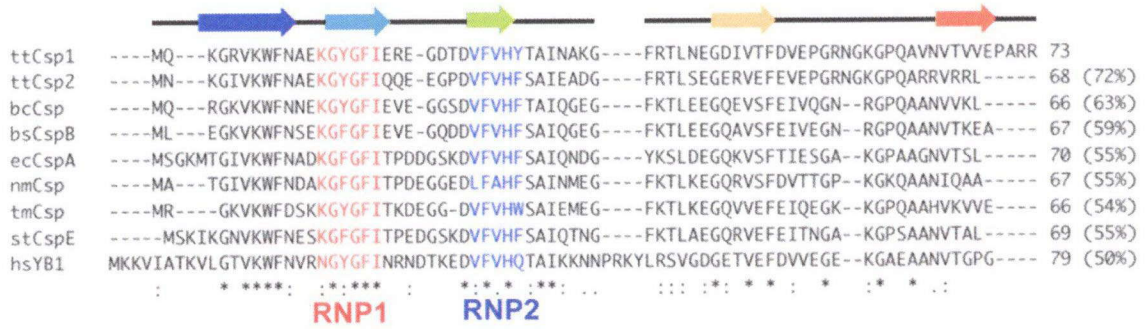
**Model statistics**

R.m.s. deviations from ideal values

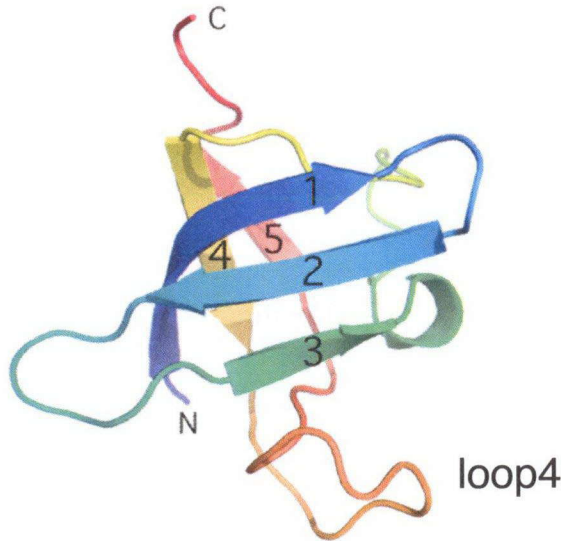
Bond lengths (Å)	0.028 Å
Bond angle (deg)	2.1°
Mean <i>B</i> -factor (Å <sup>2</sup> )	16.9 Å <sup>2</sup>
Ramachandran plot (%)	
Most favored	86.4%
Additional allowed	13.6%
Generously allowed	0
Disallowed	0

<sup>a</sup> Values in parentheses correspond to the reflections observed in the highest resolution shell.<sup>b</sup>  $R_{\text{merge}} = \frac{\sum_{hkl} \sum_i |I_{hkl} - \langle I_{hkl} \rangle|}{\sum_{hkl} \sum_i I_{hkl}}$ , where  $I$  is the observed intensity and  $\langle I \rangle$  is the averaged intensity for multiple measurement.

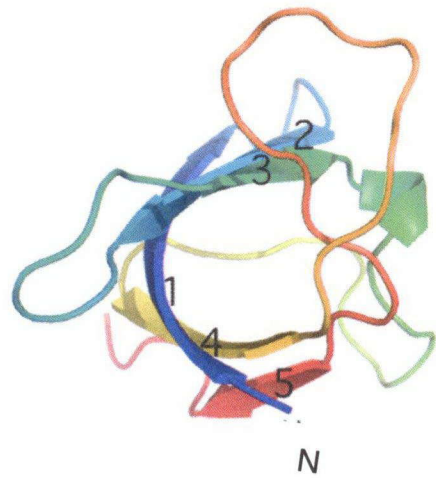
A



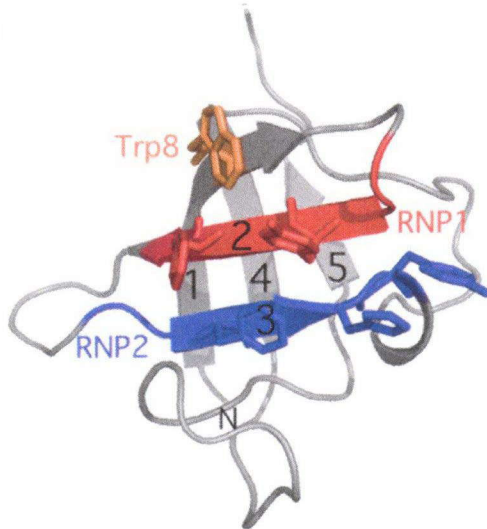
B



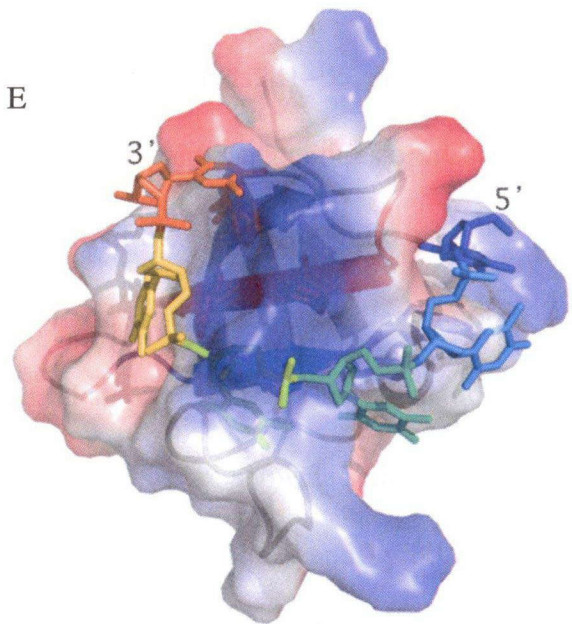
C

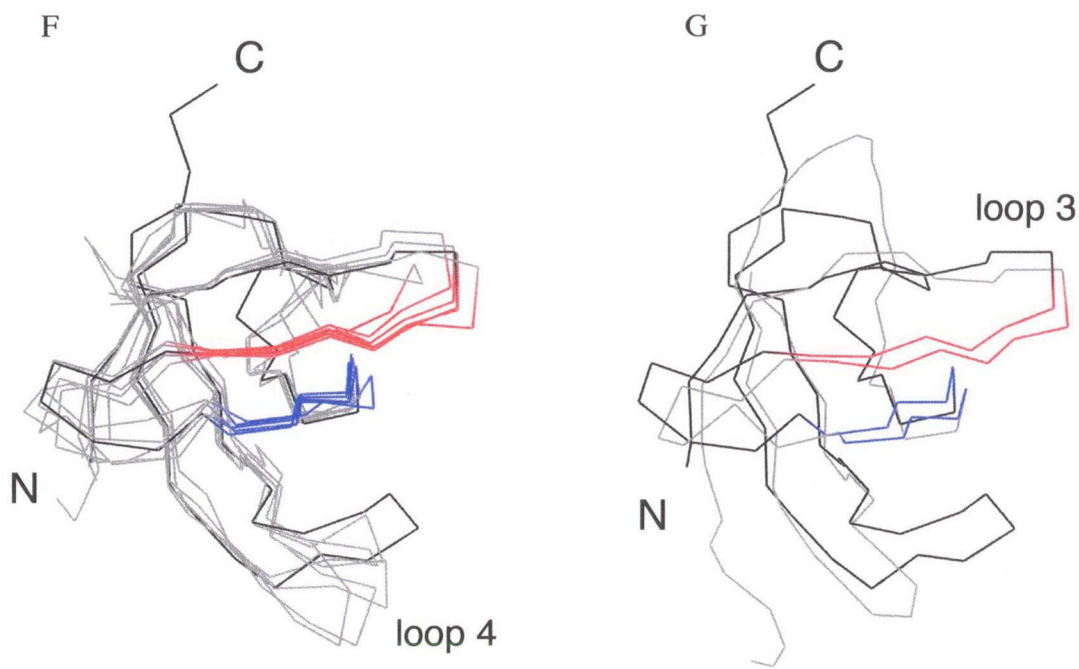


D



E





**Figure 5-1. Overall structure of *ttCsp1* and sequence alignment of Csps**

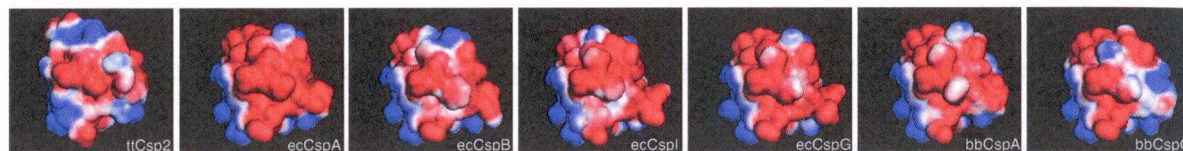
(A) Sequence alignment of 8 Csps and the CSD of hsYB1 using the ClustalW2 program (EBI web server: <http://www.ebi.ac.uk/tools/clustalw2>). Identical and homologous residues are marked with asterisks and colons, respectively. The RNP1 and RNP2 motifs are designated by red and blue characters, respectively. The  $\beta$ -strands,  $\beta_1$ – $\beta_5$ , are indicated by arrows. Values in parentheses correspond to the pairwise identities of *ttCsp1* with Csps or the CSD. (B) The schematic ribbon diagram of the overall structure of *ttCsp1*. The  $\beta$ -strands are shown as curved arrows. (C) The schematic ribbon diagram of *ttCsp1* seen from the bottom, which emphasizes the barrel structure. (D) The schematic ribbon diagram of the overall structure of *ttCsp1*. The  $\beta$ -strands are shown as curved arrows. Side chains of RNP1 (Tyr15 and Phe17, red), RNP2 (Phe27, His29 and Tyr30, blue), and Trp8 (orange) are depicted in stick form. (E) A model of the *ttCsp1*–dT6 complex. *ttCsp1* is shown along with its electron potential map on a scale from negative

(red) to positive (blue) in the same orientation as in Figure 5-1D. (F) C $\alpha$  trace of superposed *ttCsp1* and five cold shock proteins (*bcCsp*, *bsCspB*, *tmCsp*, *ecCspA* and *mnCsp*) from the same view as in Figure 5-1B. (G) C $\alpha$  trace of superposed *ttCsp1* and *hsYB1* from the same view as in Figure 5-1B. The structure of *ttCsp1* and other cold shock proteins or *hsYB1* were shown in black and gray, respectively. The RNP1 and RNP2 motifs are colored red and blue, respectively.

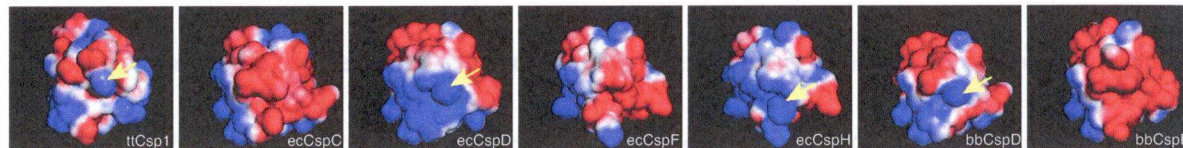
### 5-3 Comparison of surface charges of Csps

To determine the distinguishing structural features between cold-inducible (group a in Figure 5-2) and non-cold-inducible (group b) Csps, I used structure homology modeling to determine the model structures of Csps listed in Table 5-2. The surface charges of these Csps are shown in Figure 5-2. Examination of these structures reveals there are no obvious differences between the two groups of proteins. Nonetheless, it is interesting to note that in group b, four non-cold-induced Csps, including *ttCsp1*, possess a structurally conserved positively charged region. The amino acid residues in this region in *ttCsp1*, *ecCspD*, *ecCspH*, and *bbCspD* are Arg73, Lys43, Arg13, and Lys57, respectively. Hence, this region of the protein displays a similar surface charge distribution even though the amino acid sequence is not well conserved. Because this positively charged region is discrete from the nucleotide-binding site, it might be important in controlling the stabilities of complex between Csps and nucleotides or other target factors.

(a) cold-induced Csps



(b) non-cold-induced Csps



**Figure 5-2. Comparison of surface charges between *ttCsp1* and other Csps**

Surface charges of (a) cold-inducible Csps and (b) non-cold-inducible Csps are shown using the APBS1.3 program. Electrostatic potential is indicated as red (negative) or blue (positive). Among the group in (b), 4 Csps (*ttCsp1*, *ecCspD*, *ecCspH*, and *bbCspD*) have a conserved positively charged region indicated by yellow arrows.

**Table 5-2. Candidates for surface electrical charge comparison**

(a) Cold-inducible group		PDB code
<i>E. coli</i>	CspA	1MJC
<i>E. coli</i>	CspB	model
<i>E. coli</i>	CspE	model
<i>E. coli</i>	CspG	model
<i>E. coli</i>	CspI	model
<i>B. subtilis</i>	CspB	2ES2
<i>B. subtilis</i>	CspC	model
<i>B. bronchiseptica</i>	CspA	model
<i>B. bronchiseptica</i>	CspB	model
<i>B. bronchiseptica</i>	CspC	model
<i>T. thermophilus</i>	Csp2	model
(b) Non-cold-inducible group		
<i>E. coli</i>	CspC	model
<i>E. coli</i>	CspD*	model
<i>E. coli</i>	CspF	model
<i>E. coli</i>	CspH*	model
<i>B. bronchiseptica</i>	CspD*	model
<i>B. bronchiseptica</i>	CspE	model
<i>T. thermophilus</i>	Csp1*	3A0J: this study

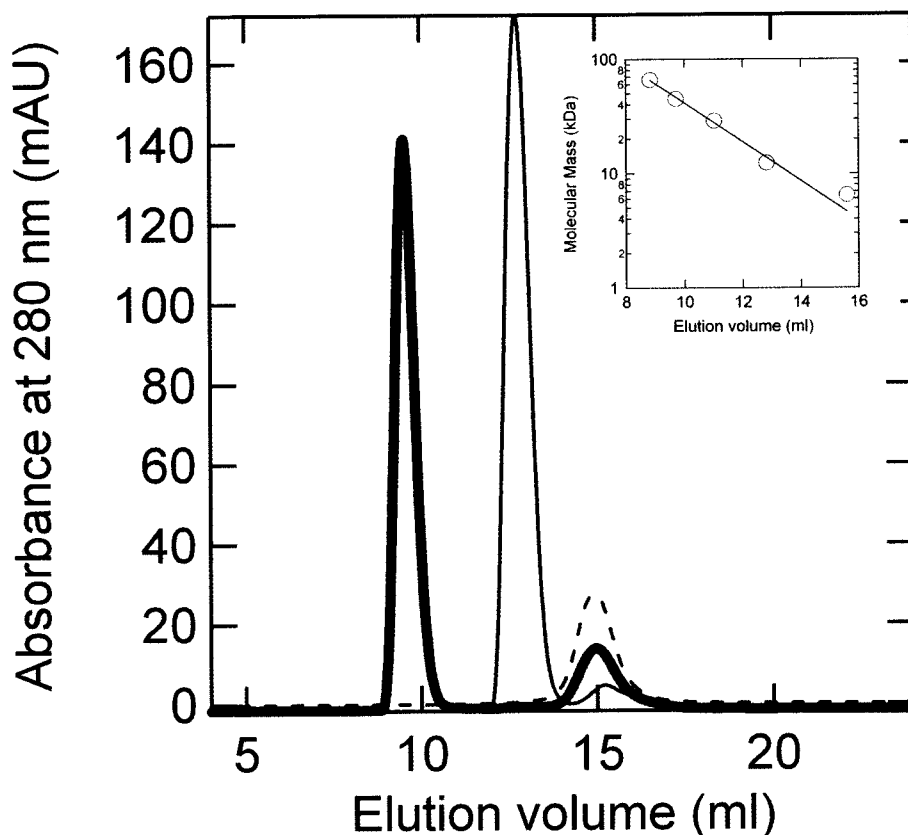
\* These Csps have positive regions.



## 6 Oligonucleotide-binding and recognition of *ttCsp1*

### 6-1. DNA-binding activity of *ttCsp1*

In order to confirm the formation of complex with *ttCsp1* and DNA, analytical size-exclusion chromatography was carried out using 7-mer oligo-dT (dT7) and 31-mer oligo-dT (dT31) (Figure 6-1). In the absence of DNA, *ttCsp1* eluted at the volume corresponding to an apparent molecular weight of 7,300, judging from the calibration curve. As the calculated molecular weight of *ttCsp1* is 8,200, *ttCsp1* was considered to exist as a monomer in solution. In the present of dT7 (M.w. 2,100) or dT31 (M.w. 9,400), the elution peaks were shifted to elution volumes corresponding to around 15,700 and 48,000, respectively. Since oligonucleotids behave on gel-filtration column as larger molecules than globular proteins, these values roughly corresponded to the complex with one *ttCsp1* and one dT7 molecules (calculated M.w. 9,400), and with five *ttCsp1* and one dT31 molecules (calculated M.w. 45,900). The ratio  $A_{260}/A_{280}$  of the elution peak in the present of dT7 was 1.05, which coincides with the calculated ratio  $A_{260}/A_{280}$ , 0.97, of the 1:1 complex with *ttCsp1* ( $\epsilon_{260}$ ,  $0.82 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ ;  $\epsilon_{280}$ ,  $1.35 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ ) and dT7 ( $\epsilon_{260}$ ,  $3.42 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ ;  $\epsilon_{280}$ ,  $2.22 \times 10^4 \text{ M}^{-1}\text{cm}^{-1}$ ). The ratio  $A_{260}/A_{280}$  of the elution peak in the present of dT31 was 1.13, which also coincides with the calculated ratio  $A_{260}/A_{280}$ , 1.14, of the complex with the 5:1 complex with *ttCsp1* and dT31 ( $\epsilon_{260}$ ,  $16.15 \times 10^4$ ;  $\epsilon_{280}$ ,  $10.92 \times 10^4$ ). These results indicate that *ttCsp1* strongly binds to ssDNA.



**Figure 6-1. Size-exclusion chromatography of *ttCsp1*.**

The dashed line, thin line and thick line represent elution profiles in the absence and the presence of dT7 and dT31, respectively. The inset shows the calibration curve. Standard proteins were used: albumin from bovine serum, 66 kDa; albumin from chicken egg, 45 kDa; carbonic anhydrase from bovine erythrocytes, 29 kDa; cytochrome c from horse heart, 12.4 kDa; aprotinin from bovine lung, 6.5 kDa.

## 6-2. Affinity for various oligonucleotides

As mentioned above, the structures of the  $\beta$ -barrel containing RNP1 and RNP2 motifs are highly conserved in cold shock proteins including *ttCsp1*. The crystal structures of *bsCspB* complexed with ssDNA (Max, K. E. *et al.*, 2006) and *bcCsp* complexed with ssDNA (Max, K. E. *et al.*, 2007) were reported. In these structures, not only the aromatic residues from RNP1 and RNP2 motifs, but also Trp8 near RNP1 motif form stacks with

nucleobases. This tryptophan residue is conserved in almost all cold shock proteins. *ttCsp1* also has the Trp8, which is the only tryptophan residue in the sequence of *ttCsp1*. In the crystal structure of *ttCsp1*, Trp8 is solvent-exposed (see Figure 5-1D).

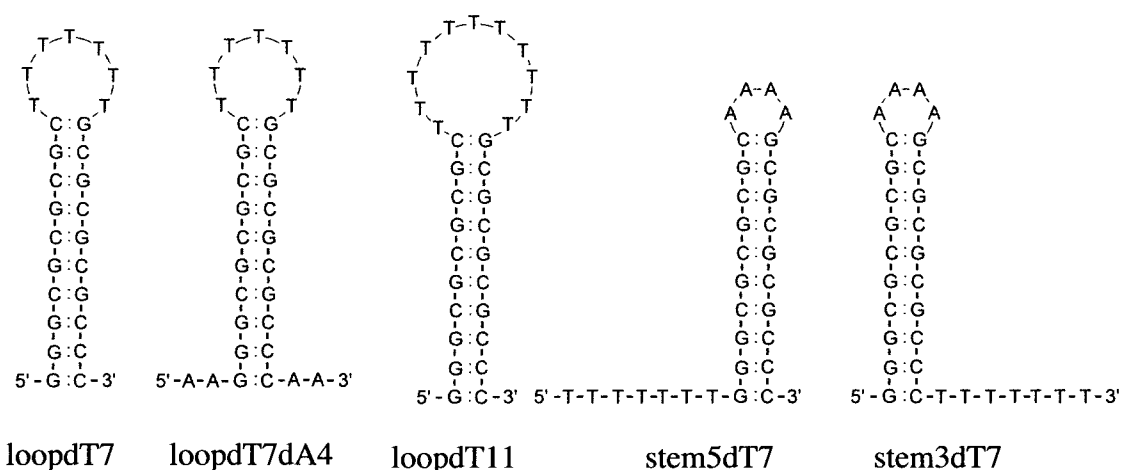
In order to analyze the binding affinity for various sequences and the secondary structures of oligonucleotides (Table 6-1, Figure 6-2), the intrinsic fluorescence from Trp8 was utilized. As shown in Figure 6-3A, the fluorescence spectrum of *ttCsp1* had an optimum intensity around 340 nm and the fluorescence intensity was almost quenched on binding of oligonucleotide, similar to the case for *bcCsp* (Max, K. E. *et al.*, 2007). These phenomena coincide with the structural feature of Trp8. The dissociation constants  $K_d$  for oligonucleotides were calculated from the decrease of fluorescence intensity to an increase of oligonucleotides (Table 6-2). Because the  $K_d$  value for dT7 was more than ten times as small as that for dA7, *ttCsp1* has a binding preference for polypyrimidines over polypurines. The  $K_d$  value for dT31 was the smallest among the oligonucleotide used, indicating more than two molecules of *ttCsp1* simultaneously can bind to dT31. From the result of analytical size exclusion chromatography, about five molecules of *ttCsp1* could bind to dT31, suggesting the binding site of *ttCsp1* accommodates about six nucleotides. It should be noted that The  $K_d$  value for stem3dT7 is the smallest among the oligonucleotides containing dT7 region. These results show not only that *ttCsp1* bind to polypyrimidines more strongly than polypurines, but also that the binding affinity of *ttCsp1* for oligonucleotide is influenced by the secondary structure around dT7 region. Specifically, *ttCsp1* might preferentially bind to unconstrained nucleotides. Furthermore, stem3dT7 bound to *ttCsp1* more tightly than stem5dT7. This suggests that the location of the stem is

important for the binding and function of *t*Csp1.

Next,  $K_d$  values of the oligo-ribonucleotide containing the U7 region with or without secondary structure were examined (Figure 6-3D and Table 6-2). Like ssDNA, the  $K_d$  value of stem3U7 was least among the other single-stranded RNAs (ssRNAs). Furthermore, loopU7 bound to *t*Csp1 more weakly than the other ssRNAs. It should be noted that *t*Csp1 bound to stem3U7 more tightly than to linearU7. This suggests that U-rich sequences possessing stem structures on their upstream regions might be the targets of *t*Csp1.

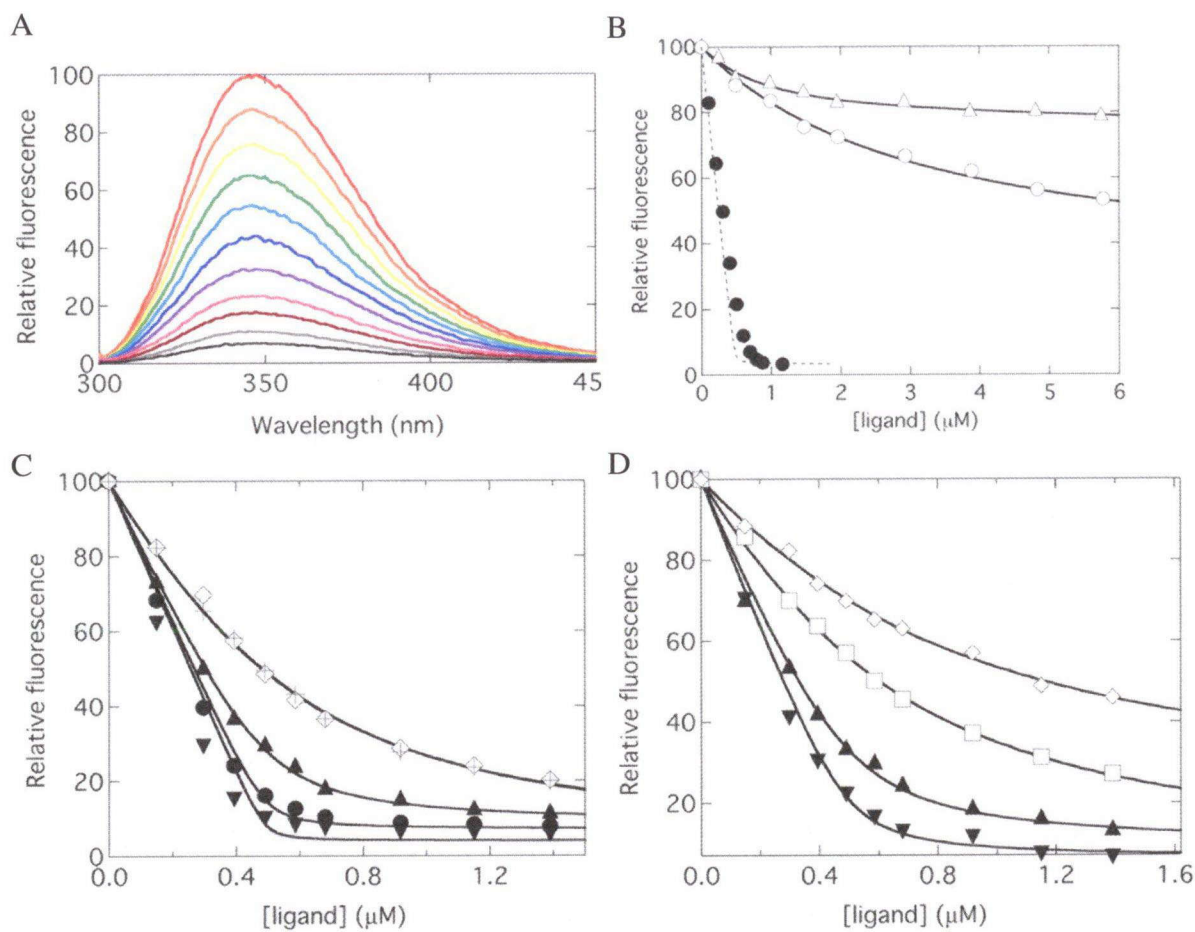
**Table 6-1. The sequences of ssDNA and ssRNA ligands.**

Name	Sequence	Length
dT7	TTTTTTT	7
dA7	AAAAAAA	7
loopdT7	CCgggCgCgCgCTTTTTTTgCgCgCgCCCgg	31
loopdT7dA4	AAgggCgCgCgCTTTTTTTgCgCgCgCCCAA	31
loopdT11	gggCgCgCgCTTTTTTTTTTTgCgCgCgCCC	31
stem5dT7	TTTTTTTgggCgCgCgCAAAAgCgCgCgCCC	31
stem3dT7	gggCgCgCgCAAAAgCgCgCgCCCTTTTTTT	31
dT31	TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT	31
dA31	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA	31
U7	UUUUUUU	7
loopU7	CCgggCgCgCgCUUUUUUgCgCgCgCCCgg	31
stem5U7	UUUUUUUgggCgCgCgCAAAAgCgCgCgCCC	31
stem3U7	gggCgCgCgCAAAAgCgCgCgCCCUUUUUU	31



**Figure 6-2. The predicted secondary structures of ssDNA**

The ligands of loopdT7, loopdT7dA4, loopdT11, stem5dT7 and stem3dT7 were prepared by incubating at 95°C for 2 min and 60°C for 10 min to form the secondary structures.



**Figure 6-3 Binding of *ttCsp1* to nucleic acids**

(A) Fluorescence spectra of *ttCsp1* in the presence of various concentrations of dT7: 0 (red), 0.1 (orange), 0.2 (yellow), 0.3 (light green), 0.4 (cyan), 0.5 (blue), 0.6 (purple), 0.7 (pink), 0.8 (brown), 0.9 (grey), and 1.0 μM (black). (B) Changes in fluorescence intensity at 350 nm: dT7 (filled circles), dA7 (open circles), and dT4 (open triangles). The dashed line was calculated from the  $K_d$ , which was determined by the competition experiment. (C) Change in fluorescence intensity at 350 nm in the presence of 10 μM dT4 as a competitor: dT7 (filled circles), loopdT7 (open diamonds), loopdT11 (crosses), stem5dT7 (filled triangles), and stem3dT7 (filled inverse triangles). (D) Change in fluorescence intensity at 350 nm in the presence of 10 μM dT4 as a competitor: U7 (open squares), loopU7 (open diamonds), stem5U7 (filled triangles), and stem3U7 (filled inverse triangles).

**Table 6-2. The dissociation constants of *tCsp1* for ssDNA and ssRNA ligands**

ligand	length	$K_d$ (nM)	method
dT4	4	2800	direct
dA7	4	590	direct
dT7	7	0.91	competition
loopdT7	31	57	competition
loopdT11	31	55	competition
stem5dT7	31	6.9	competition
stem3dT7	31	0.27	competition
U7	7	80	competition
loopU7	31	130	competition
stem5U7	31	11	competition
stem3U7	31	3.6	competition

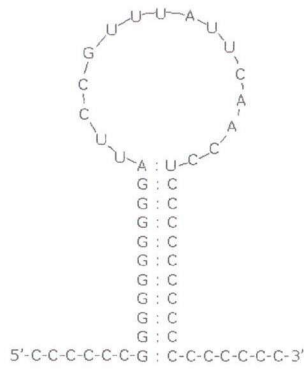
The description of “direct” in the method column represents the fluorescence titration of *tCsp1* with a ligand, and “competition” represents the titration in the presence of dT4 (see *Materials and Methods* for details).

<sup>a</sup> The dissociation constant for dT31 was undetectable by using this method, because of the too strong affinity above the detection limit of the fluorescence spectrophotometer.

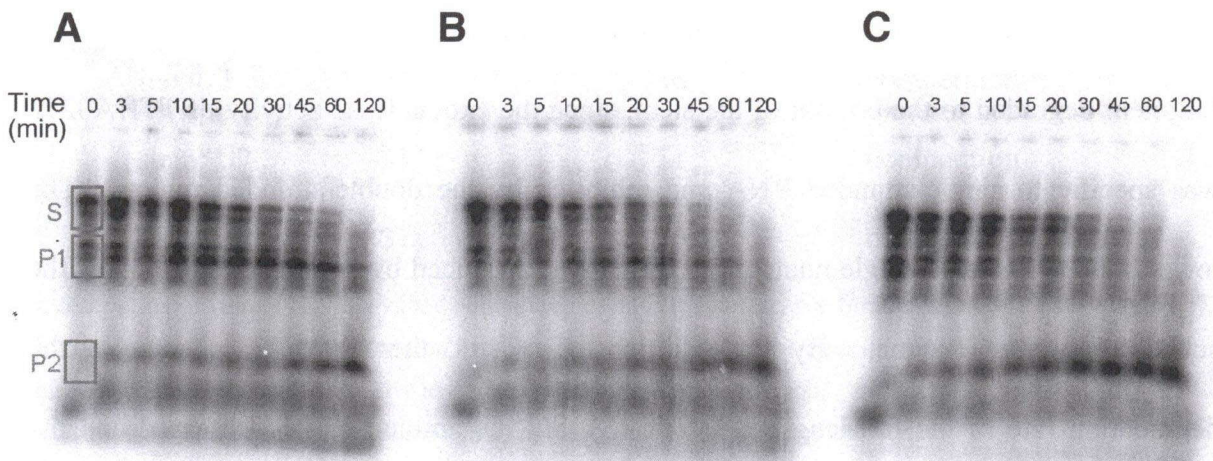
## 7. Effect of *ttCsp1* on RNase activity

Now I know that *ttCsp1* can work both on transcriptional and translational processes. Some of gene expressions under the cold condition might be controlled by changing the amounts of mRNAs. To test the hypothesis that *ttCsp1* helps RNase degrade mRNAs, I measured exonuclease activity of a RNase in the absence and presence of *ttCsp1* *in vitro*. The employed RNase was TTHA0252 of *T. thermophilus* HB8, which has single-strand-specific 5'-3' exonuclease activity (Ishikawa, H. *et al.*, 2006). The RNA substrate employed was 50-mer hairpin RNA (h-RNA) as shown in Figure 7-1. When TTHA0252 was reacted with h-RNA, two main products were observed on a gel (Figure 7-2). Among these two bands, the upper band (P1) was likely to correspond to the fragment degraded to the bottom of the stem, since the exonuclease activity of TTHA0252 was specific to single-stranded RNA and inhibited by the double-stranded region. The lower band (P2) was a single nucleotide, which was produced by complete digestion of the substrate by the enzyme. By the addition of *ttCsp1*, there was no change in the degradation rate of the full-length substrate (S), but the amount of P1 decreased and the amount of P2 increased (Figure 7-3). These results suggest that *ttCsp1* did not affect the nuclease activity of RNase, but melted the stem structure of the substrate to help RNase to proceed through the stem-loop. The effect of *ttCsp1* on RNA degradation dependent on melting of the secondary structure may be important for adaptation to stress conditions.



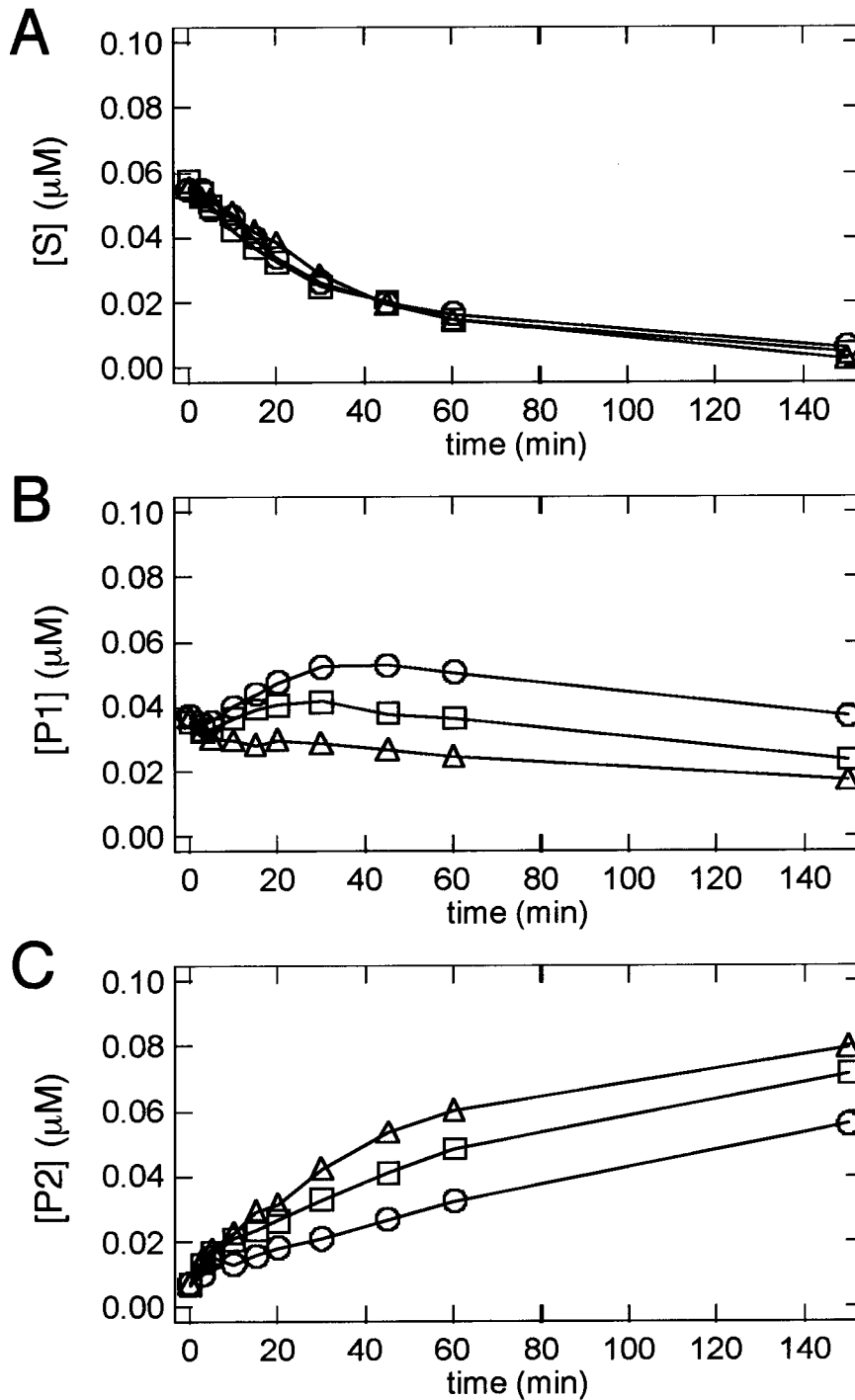


**Figure 7-1. Sequence and predicted secondary structure of h-RNA.**



**Figure 7-2. Degradation of h-RNA with 0  $\mu$ M (A), 0.2  $\mu$ M (B) and 23  $\mu$ M (C) *tCsp1*.**

The substrate h-RNA was radiolabeled at 3'-end. The assay was performed at 37°C.



**Figure 7-3. Effect of *tCsp1* on RNase activity to hairpin RNA.**

The amounts of band S (A), P1 (B) and P2 (C) in the presence of 0  $\mu\text{M}$  (circles), 0.2  $\mu\text{M}$  (squares) and 23  $\mu\text{M}$  (triangles) *tCsp1* were plotted against reaction time.

## 8. Working mechanism of *ttCsp1* under optimal and cold condition

Under optimal growth conditions there were no differences in the growth curves (Figure 1-1) and gene expression profiles of wild-type and  $\Delta ttcsp1$  cells. These results indicate *ttCsp1* does not significantly contribute to the transcriptional regulation at the physiological temperature (70°C). However, it is reported that single-gene disruptants of *ttcsp1* exhibit similar growth patterns to wild-type cells and  $\Delta ttcsp2$  was not lethal at 45°C. Moreover, the double disruptant (*ttcsp1* and *ttcsp2*) exhibited a lower growth rate by comparison to the other three strains (wild-type and disruptants of *ttcsp1* or *ttcsp2* strains) at 45°C. These results suggest that *ttCsp1* can compensate for the functions of *ttCsp2* at low temperature. There was a time lag between the mRNA (30 s) and protein expression (10 min) of *ttCsp2* after a temperature drop (Mega, R. *et al.*, 2010). In addition, the microarray data show that there were up-regulated genes at 45°C in only wild-type cells. These results suggest that *ttCsp1* regulates gene expression by acting as a transcriptional anti-terminator until *ttCsp2* is expressed at low temperature. Indeed, constitutively expressed Csps, *ecCspC* and *ecCspE*, have been shown to operate as transcriptional anti-terminators in *E. coli* (Bae, W. *et al.*, 2000, Phadtare, S. *et al.*, 2007). It is, *ttCsp1* can work also as a transcriptional regulator under the cold condition. The influences of *ttCsp1* on RNase activities support the existence of the transcriptional regulation system which *ttCsp1* is involved in.

From the comparison of results from microarray and proteome, about more than half of influenced proteins by *ttcsp1* deletion, significant changes of gene expression were

detected only in translational level (see chapter 4 groupB). In addition, without *ttCsp1*, any changes of transcriptional levels were not detected under the optimal growth condition although the amount of some proteins (translational level) were increased or decreased. *In vitro* experiments revealed that *ttCsp1* binds to single-stranded oligonucleotides with low specificity to bases and secondary structures of oligonucleotides, although these results were based on limited variation of nucleotides. Nevertheless, it is possible to suppose that the states of nucleotide affect binding to *ttCsp1*. From the obtained results, I focused on the working mechanism of *ttCsp1* in translational level under the optimal condition in this section. I propose the following models.

#### 8-1 Translation of some proteins are inhibited by *ttCsp1*.

As shown in Figure 8-1A, where translation is inhibited by *ttCsp1*, the mRNA has a U-rich region and the 5' region is predicted to form a secondary structure. *ttCsp1* binds to the U-rich region and a large stem and loop are formed (Figure 8-1B). Furthermore, *ttCsp1* interacts with the sugar-phosphate backbone of the stem, stabilizing the complex (Figure 8-1C). This stable complex inhibits translation from the mRNA. In the absence of *ttCsp1*, no large stem and loop are formed, although, two small stems and loops that do not inhibit the translation of the mRNA are generated (Figure 8-1D).

#### 8-2 *ttCsp1* stimulates the translation of some proteins.

Where translation is stimulated by *ttCsp1*, the mRNA has a U-rich region that is predicted to form a secondary structure (Figure 8-2A). *ttCsp1* binds to the U-rich region

and prevents it from forming a secondary structure (Figure 8-2B). In the absence of *tCsp1*, a large stem and loop are formed where translation arrest readily occurs (Figure 8-2C). This is a very sophisticated control system because mRNA conformations are in flux and easily altered by intracellular and/or extracellular environmental conditions; thus, *tCsp1* can function as both an enhancer and inhibitor of translation.

In conclusion, through a combination of transcriptome and proteome analyses, I have demonstrated that the control systems of transcription and translation are even more complex than originally thought, even in prokaryotes. I suggest that non-cold-inducible Csps are candidates for translational controllers. Some, but not all, non-cold-inducible Csps may play a role in monitoring stress and fine tuning cellular processes in order to adapt to changing environmental conditions. In addition to temperature, the structure of mRNA can be influenced by pH, osmotic pressure and salt concentrations. Thus, I conclude that Csp family proteins may respond to different stress factors by alterations to their nucleotide binding affinities according to the structures of the target nucleotides. Moreover, the electric potential of Csps around the RNP motifs may be an important factor in determining their distinct functions. Consequently, the control mechanism for Csps is extremely flexible. The flexibility of the function is important for the fine tuning against subtle changes of conditions.

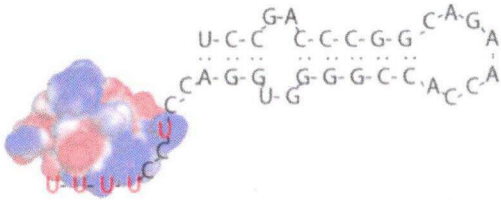
A

781 -

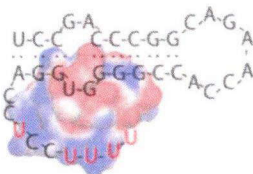
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CGGGTGAGCGTCCTCTCCGACCGGCAGAACCACCGGGTGGACCTCCTTTTGGAGGAAGGC  
CGGATCCTCCTCTCCGCCGAGGGGGACTACGGCAAGGGGCAGGAGGAGGTGCCCGCCAG
```

- 960

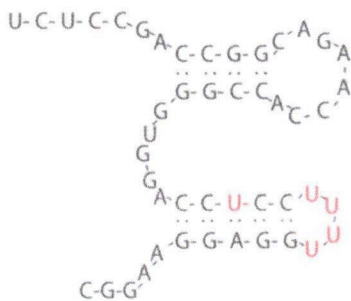
B



C



D



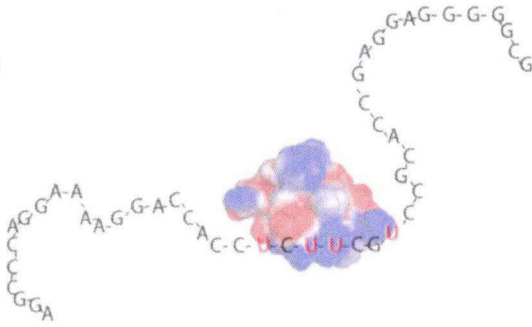
**Figure 8-1. The gene down-regulated in  $\Delta$ *ttcsp1*, TTHA0001**

(A) Nucleotide sequence of TTHA0001. The dT(U)-rich region is indicated by a box. Underline indicates the region predicted to form the secondary structure. The secondary structure of the dT(U)-rich box was predicted using vsfold5 (Dawson, W. *et al.*, 2007) (B) Binding of *ttCsp1* to the dT-rich region and formation of a large stem and loop. (C) Stabilization of the *ttCsp1* complex with the target region. (D) Formation of 2 small stems and loops that do not inhibit the translation of the mRNA in the absence of *ttCsp1*.

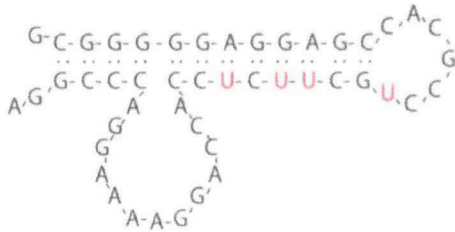
A

421 -  
GAGGAGCTCCGCCAGCGCTACGCCGAGCTCGTCCCCGTGGAGCGGGAGGCCAGGAAAAG  
GACCACCTC TTCGTCCGCACCGAGGAGGGGGCGGAGTTCCCCATTGACCTCGCTAAAGCC  
CTTCCCCACGTGCGGGAGGCCCTCCTCGGCAAGAAGGCGGGGGACGTGGTCATGGTCCCC  
- 600

B



C



**Figure 8-2. The gene up-regulated in  $\Delta ttcsp1$ , TTHA0614**

(A) Nucleotide sequence of TTHA0614. The dT(U)-rich region is indicated by a box. Underline indicates the region predicted to form the secondary structure. (B) Binding of *ttCsp1* to the dT(U)-rich region. (C) Formation of a large stem and loop in the absence of *ttCsp1*.

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Tanaka, T., Mega, R., Kim, K., Shinkai, A., Masui, R., Kuramitsu, S. and Nakagawa, N.  
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