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Heterologous Expression of Thermolabile Proteins Enhances Thermotolerance in *Escherichia coli*

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Abstract

Heat shock proteins (HSPs) play important roles in the mechanism of cellular protection against various environmental stresses. It is well known that accumulation of misfolded proteins in a cell triggers the HSPs expression in prokaryotes as well as eukaryotes. In this study, we hetero- logously expressed two proteins in *E. coli*, namely, citrate synthase (*Cp*CSY) and malate dehydrogenase (*Cp*MDH) from a psychrophilic bacterium *Colwellia psychrerythraea* 34H (optimal growth temperature 8˚C). Our analyses using circular dichromism along with temperature-dependant enzyme activities measured in purified or direct cell extracts confirmed that the *Cp*CSY and *Cp*MDH are thermolabile and present in misfolded form even at physiological growth temperature. We observed that the cellular levels of HSPs, both GroEL and DnaK cheperonins were increased. Similarly, higher levels were observed for sigma factor σ^{32} which is specific to heat-shock protein expression. These results suggest that the misfolded-thermolabile proteins expressed in *E. coli* induced the heat shock response. Furthermore, heat treatment (53˚C) to wild type *E. coli* noticeably delayed their growth recovery but cells expressing *Cp*CSY and *Cp*MDH recovered their growth much faster than that of wild type *E. coli*. This reveals that the HSPs expressed in response to misfolded-thermolabile proteins protected *E. coli* against heat-induced damage. This novel approach may be a useful tool for investigating stress-tolerance mechanisms of *E. coli*.

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Keywords

Heat Shock Proteins, σ^{32}, Psychrophilic Proteins, Thermotolerance

1. Introduction

The accumulation of unfolded or misfolded proteins is one of the major factors leading to increased expression of highly conserved proteins called heat shock proteins (HSPs). The HSPs include the molecular chaperones, such as GroEL/GroES and DnaK, which help cellular proteins to maintain the proper folding required for function [1] [2]. It also includes some proteases, such as ClpAP, ClpXP, and FtsH, which degrade the unfolded proteins [3]. In *E. coli*, HSP expression is positively controlled by σ^{32}, the alternative subunit of RNA polymerase specific to the heat-shock promoter [4]. Under physiological conditions, DnaK chaperone system traps σ^{32} to mediate its degradation by proteases, mainly FtsH, an AAA protease associated with the inner membrane [5]-[7]. On the other hand, under stress conditions, the DnaK system interacts with the unfolded proteins, and releases σ^{32}. As a result, σ^{32} activate the transcription of several HSP genes. A similar model is also proposed in eukaryotes, for example, heat shock response mediated by a transcriptional factor, Hsf1 [8]-[11], and unfolded protein response in the endoplasmic reticulum [12].

Psychrophiles can inhabit at lower temperatures, generally in the range of 0˚C - 15˚C [13]. The transcription of HSP genes in psychrophilic bacterium such as *Colwellia maris* ABE-1 is induced at much lower temperatures, such as 20˚C, than those of mesophilic ones [14]-[16]. Therefore, certain proteins of the psychrophiles may be in misfolded state at physiological growth temperature for most mesophiles. Adaptation of enzymes to cold environments should be essential for the survival and growth of psychrophilic bacteria under cold environmental conditions. Although cold-adapted enzymes exhibit high specific activities at low temperature, they also display pronounced thermolability compared with their mesophilic and thermophilic counterparts. For example, isocitrate lyase of a psychrophilic bacterium, *Colwellia psychrerythraea* NRC 1004, showed the maximum activity at 25˚C and was completely inactivated by incubation even at 30˚C for 2 min [17]. Similar results are reported for citrate synthase from an Antarctic bacterial strain, DS2-3R [18], and for malate dehydrogenase from the psychrophilic bacterium, *Flavobacterium frigidimaris* KUC-1 [19].

The acquisition of thermotolerance by an organism is correlated with the elevated expression of HSPs. Owing to their protective functions under high-temperature conditions; overexpression of HSPs has been used as a promising technique to improve the thermotolerance of transgenic organisms [20]-[23]. To date, most of these trials have been performed using one or two HSP genes. However, if we consider that several types of HSPs function synergistically in living cells, their multiple expressions by gene manipulation should lead to further improvement compared with the expression of specific HSPs individually.

Given that psychrophilic proteins are misfolded at physiological growth temperatures of *E. coli*, heterologous expression of psychrophilic proteins would increase the expression level of HSPs in transformed cells. Taking these observations together, the thermolabile nature of psychrophilic proteins could be utilized as a signal to induce the synthesis of HSPs at physiological growth temperatures of *E. coli* that could enhance thermotolerance. Such cells having higher HSP levels can tolerate stress and recovery should be quicker than wild type. In the present study, we transformed *E. coli* cells with two genes encoding citrate synthase (*Cp*CSY) and malate dehydrogenase (*Cp*MDH) from a psychrophilic bacterium *Colwellia psychrerythraea* 34H and also their analogues native to *E. coli*. We investigated the change in cellular levels of HSPs, such as GroEL and DnaK; as well as an alternative sigma factor of RNA polymerase σ^{32}. These three factors were analyzed because ordered network between GroE and DnaK is essential for tightly regulating σ^{32} activities which is central to the expression of HSP genes [24]. We found that the expression of these psychrophilic proteins in misfolded/unfolded state enhanced thermotolerance of *E. coli* cells due to altered cellular levels of HSPs.

2. Materials and Methods

2.1. Bacteria, Culture Conditions, and DNA Preparation

The psychrophilic bacterium *Colwellia psychrerythraea* strain 34H was obtained from American Type Culture

Collection (Manassas, USA) grown to stationary phase in Marine Broth (Difco, Lawrence, KS, USA) at 8˚C. *E. coli* strains JM109 and BL21 purchased from Takara (Japan) were used for the propagation of plasmids and the heterologous expression of recombinant proteins, respectively. Unless otherwise stated, these *E. coli* strains were grown at 37˚C with vigorous shaking in LB medium supplemented with 50 μg·ml^{-1} ampicillin when required. Genomic DNAs of *C. psychrerythraea* and *E. coli* were prepared by methods described previously [14].

2.2. Plasmid Construction

Restriction endonucleases were obtained from New England Biolabs (Beverly, MA, USA) and DNA-modifying enzymes for plasmid construction were from Takara Shuzo (Kyoto, Japan). The coding regions of genes for MDH and CSY of *C. psychrerythraea* and *E. coli* were amplified by PCR with KOD plus DNA polymerase (Toyobo, Osaka, Japan) and corresponding PCR primers (**Table 1**). The PCR products were digested with *Nde*I and *Xho*I. The DNA fragments were cloned into the corresponding sites of pET-21b vector (Novagen, Darmstadt, Germany). The resultant plasmids for *C. psychrerythraea* enzymes were designated as p-CpMDH and p-CpCSY, and those for *E. coli* enzymes were designated as p-EcMDH and p-EcCSY, respectively. Sequences of all constructs were confirmed by DNA sequencing using an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.3. Heterologous Expression and Purification of His-Tagged Proteins in *E. coli*

The plasmids described above were used to transform *E. coli* BL21, and the resultant transformants were grown at 37˚C until OD_{600} of the culture reached 0.5. The heterologous expression of His-tagged recombinant proteins was induced by the addition of 0.1 mM IPTG at 30˚C for MDHs and at 20˚C for CSYs. Cells were harvested and suspended in 5 ml of a solution containing 20 mM phosphate buffer saline (PBS, pH 7.4) and 500 mM NaCl (buffer A). After sonication, soluble cell extracts were obtained by centrifugation (15,000 rpm) at 4˚C for 30 min, and applied to a 1-ml HiTrap chelating column (GE Healthcare, Little Chalfont, UK) that had been equilibrated with buffer A containing 10 mM imidazole. The column was washed with buffer A containing 40 mM imidazole, and His-tagged proteins were then eluted with buffer A containing 500 mM imidazole. Eluted fractions were applied to a 5-mL HiTrap desalting column (GE Healthcare) that had been equilibrated with a solution containing 20 mM Hepes/KOH (pH 7.5), 50 mM NaCl, and 10% glycerol, and purified His-tagged proteins were stored at −80˚C until use. Purity of proteins was checked by SDS-PAGE and protein concentration was determined using a Protein Assay Kit (Bio-Rad, Hercules, CA, USA) with BSA as the standard.

2.4. Enzyme Assay

The CSY activity was assayed by measuring the increase of absorbance at 412 nm with an Ultrospec 3000 spectrophotometer (GE Healthcare) using method of [25]. The assay buffer (100 μl) contained 110 mM Tris/HCl (pH 8.0), 2.5 mM EDTA, 0.4 mM DTNB, 0.6 mM oxaloacetic acid, 0.2 mM acetyl-CoA, and an appropriate amount of enzyme. The reaction was started by addition of acetyl-CoA into the solution.

Table 1. Oligonucleotide primers used for PCR amplification.

Protein	Primer type	Nucleotide sequence	Length (bp)
*Cp*MDH	Forward	5’-CATATGAAAGTAGCTGTTTTAGG-3’	23
	Reverse	5’-CTCGAGGCTAGCCATAAAATCAA-3’	23
*Cp*CSY	Forward	5’-CATATGGCTGAATCAAAAGCCA-3’	23
	Reverse	5’-CTCGAGTCTTTTATTTAATGGTA-3’	23
*Ec*MDH	Forward	5’-CATATGAAAGTCGCAGTCCTC-3’	21
	Reverse	5’-CTCGAGCTTATTAACGAACTCTT-3’	23
*Ec*CSY	Forward	5’-CATATGGCTGATACAAAAGCAA-3’	22
	Reverse	5’-CTCGAGACGCTTGATATCGCTTT-3’	23

The MDH activity was assayed by measuring the decrease of absorbance at 340 nm due to the conversion from NADH to NAD^+ with a UV1800 spectrophotometer (Hitachi, Tokyo, Japan). The reaction solution contained 100 mM Tris-HCl (pH 7.8), 1 mM DTT, 0.2 mM NADH, 1 mM oxaloacetic acid, and an appropriate amount of enzyme in a final volume of 800 μl. The reaction was started by the addition of NADH into the solution.

For the assay of enzyme activity in cell extracts, cells heterologously expressing recombinant proteins were disrupted by sonication and the resultant homogenate was centrifuged at 15,000 rpm for 20 min. The enzymatic activity was determined using soluble fraction as described above.

2.5. Circular Dichroism

Protein concentrations were determined by measuring the optical absorption at 280 nm. A circular dichroism (CD) spectra of recombinant proteins were obtained with aJ-800 spectrometer (JASCO), equipped with a Peltier thermo controller and using a path-length of 0.1 cm. From 8˚C to 50˚C, protein sample of 8 μMin Tris-HCl buffer (pH 7.4) was placed in the quartz cell (1 mm thickness) and 32 scans were averaged. The molar extinction coefficients were determined according to the method of [26].

2.6. Western Blot Analysis

Cell extracts prepared from each recombinant *E. coli* cells, as described above, were solubilized and equal amount of processed sample was resolved on 12.5% SDS-PAGE. The proteins were transferred onto a PVDF membrane, Hybo resolved on nd-P (GE Healthcare). HSPs were detected with antibodies against GroEL (Assay Pro, St. Charles, MO, USA), DnaK (Enzo Life Sciences, Farmingdale, NY, USA), and σ^{32} (Neo Clone, Madison, WI, USA), using the ECL prime Western Blotting Detection system (GE Healthcare).Using ImageJ program, we measured relative levels of GroEL, DnaK and σ^{32} by densitometry analysis of the same area from each lane representing wild type and respective recombinant proteins.

2.7. Effects of High Temperature Treatment on Growth of *E. coli*

E. coli cells were cultivated at 30˚C until OD_{600} of the culture reached 0.5. The heterologous expression of recombinant proteins was induced by incubation with 0.1 mM IPTG for 2 h at 30˚C for MDHs and at 20˚C for CSYs. The concentration of these cultures was adjusted as OD_{600} = 0.5, and then incubated at high temperature of 53˚C for 15 min. For the dot assay, each culture was diluted serially (1:10, 1:100, 1:1000, and 1:10000), and 2.5 μl of each sample was spotted onto LB agar plates and incubated at 30˚C for cells heterologously expressing MDHs and at 20˚C for cells heterologously expressing CSYs.

For the growth curve assay, cells were grown and expression of recombinant proteins was induced as described above. After treatment at high temperature of 53˚C for 15 min, cells were grown in LB medium at 20˚C or 30˚C and absorbance at 600 nm was measured at regular time interval.

3. Results

3.1. Thermolability of *Cp*CSY and *Cp*MDH

In order to confirm whether *Cp*CSY and *Cp*MDH are thermolabile proteins, we first compared the temperature-dependent enzymatic activity of recombinant *Cp*CSY and *Cp*MDH with that of CSY and MDH from *E. coli* (*Ec*CSY and *Ec*MDH). Overexpressed proteins were purified by Ni-affinity chromatography and purity was analyzed by CBB staining after SDS-PAGE separation (**Figure 1**).

These purified proteins were incubated at various temperatures (8˚C, 15˚C, 25˚C, 30˚C, 40˚C and 50˚C) for 1 h and then their residual enzymatic activity was determined (**Figure 2(a)**). The residual enzymatic activity of *Cp*CSY, *Cp*MDH, *Ec*CSY and *Ec*MDH was reduced to a half level at ~16˚C, ~22˚C, ~43˚C, and ~50˚C respectively. These results indicate that *Cp*CSY and *Cp*MDH were more thermolabile than *Ec*CSY and *Ec*MDH respectively; *i.e.* the temperatures causing inactivation of *C. psychrerythraea* enzymes under *in vitro* conditions were much lower than those of *E. coli* enzymes. Also, among the two psychrophilic proteins, *Cp*CSY was found to be more thermolabile than *Ec*MDH.

The CD spectrum for each recombinant protein (**Figure 2(b)**) is characterized by two negative peaks at 207 nm

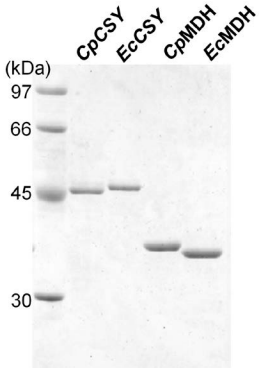


Figure 1. The SDS-PAGE analysis of purified CSY and MDH. Each protein (2 μg) was resolved on 12.5% SDS-PAGE and visualized by CBB-R250 staining. Lane 1, protein marker.

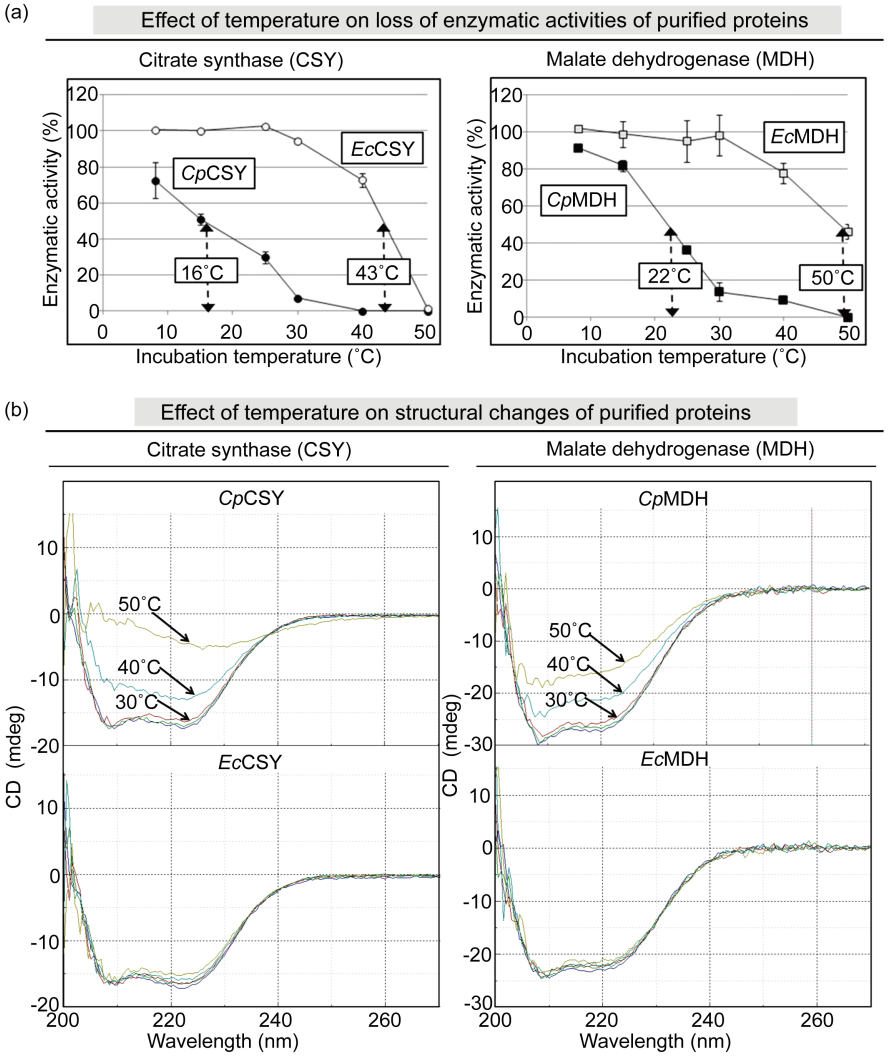


Figure 2. (a) Thermolability of recombinant *Cp*CSY, *Cp*MDH, *Ec*CSY and *Ec*MDH. Proteins overexpressed and purified from *Escherichia coli*. Each protein was incubated for 1 h at designated temperatures and then rapidly cooled on ice. The residual activity was determined as described in Materials and Methods. Symbols: *Cp*CSY (*filled circle*), *Ec*CSY (*gray circle*), *Cp*MDH (*filled square*), and *Ec*MDH (*gray square*). Residual activity was calculated in terms of percentage compare to that before the incubation. (b) Representative spectra of circular dichromism measurements for all four proteins.

and 222 nm. This data is indicative of the presence of a mixture of secondary-structural features such as α-helix, β-sheet, turn and unordered form. The CD measurements of recombinant *E. coli* proteins (*Ec*CSY and *Ec*MDH) at different temperatures showed no change in the negative peak indicating higher temperatures induced no conformational changes within the *Ec*CSY and *Ec*MDH. On the other hand, recombinant proteins from psychrophilic bacterium (*Cp*CSY and *Cp*MDH) overexpressed in *E. coli* and then purified, showed distinctive changes in the negative peak at higher temperatures. Slight change in negative peak was observed at 30˚C. The CD spectra at 50˚C suggested that almost whole amount of *Cp*CSY and *Cp*MDH were present in unordered form (*i.e.* reducedα-helices and β-sheet contents). This data showing loss of structural features at higher temperatures is in agreement with the reduced enzymatic activities of *Cp*CSY and *Cp*MDH (**Figure 2(a)**) and certifies that both the proteins *i.e.* *Cp*CSY and *Cp*MDH are thermolabile. The temperature conditions at 20˚C for CSY-expressing cells, and 30˚C for the MDH-expressing cells, were used for expression of recombinant CSY and MDH in further study and referred as permissive conditions.

We also examined the thermolabile nature of *Cp*CSY and *Cp*MDH by measuring relative enzymatic activity directly in crude cell extracts from cultures grown at 20˚C and 30˚C respectively (**Figure 3(a)**). The enzymatic activities showed 30% and 50% reduced activity for *Cp*CSY and *Cp*MDH compared to those corresponding *Ec*CSY- and *Ec*MDH-expressing cells respectively. Since the cellular expression levels of recombinant proteins

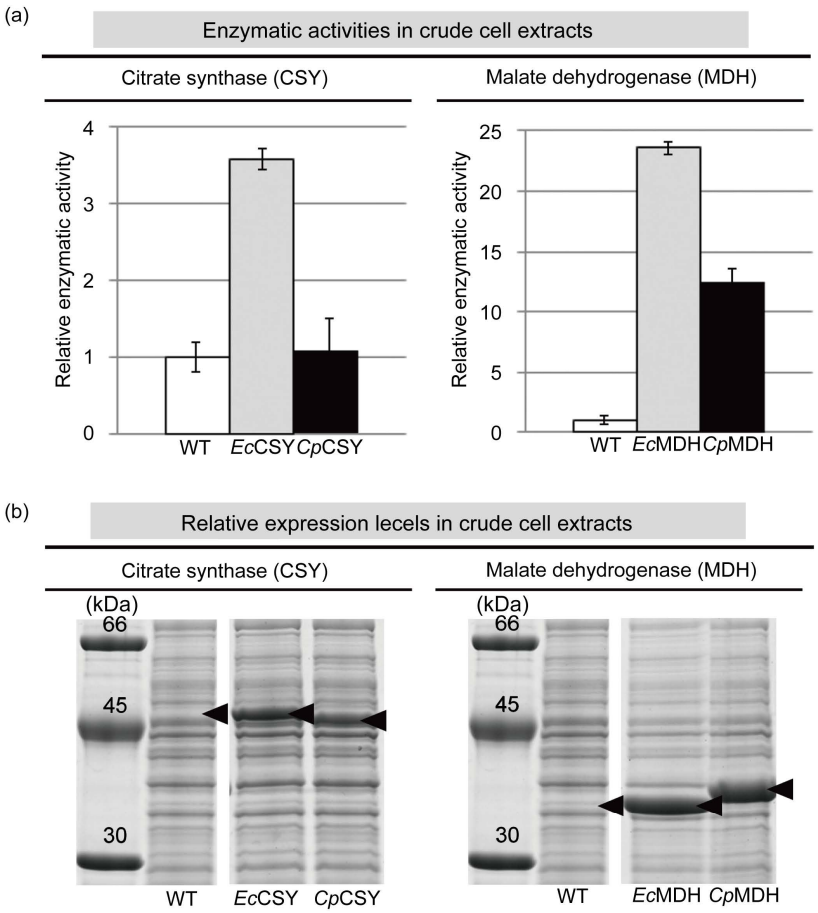


Figure 3. (a) Analysis of enzymatic activities measured directly in cell extracts. Soluble fraction of cell extracts from *E. coli* cells harboring only the pET21-b vector (white bars), cells over-expressing *Ec*CSY or *Ec*MDH (gray bars), and cells over-expressing *Cp*CSY or *Cp*MDH (black bars) were used for enzyme assay. Relative activities of CSYs and MDHs against respective vector control (pET21-b, abbreviated as WT, wild type) were plotted in graph. (b) The cell extracts were also processed for analysis by SDS-PAGE (12.5% of polyacrylamide-gel percentage) and visualized by CBB staining. Arrowheads indicate the bands of corresponding over-expressed proteins. The MW of each band in protein marker is indicated above the respective bands. All samples were electrophoresed in the same gel and the SDS-PAGE data are rearranged to facilitate better comparison between amounts of expressed proteins.

(*Cp*CSY compared to *Ec*CSY or *Cp*MDH compared *Ec*MDH) were almost the same in *E. coli* (**Figure 3(b)**), lower enzymatic activities in *Cp*CSY- and *Cp*MDH-expressing cells likely to be due to the misfolded-psychrophilic proteins as observed in CD measurements or *in vitro* enzymatic assay using purified proteins.

3.2. Heterologous Expression of *Cp*CSY and *Cp*MDH in *E. coli* Cells Enhanced the Expression of Heat Shock Proteins

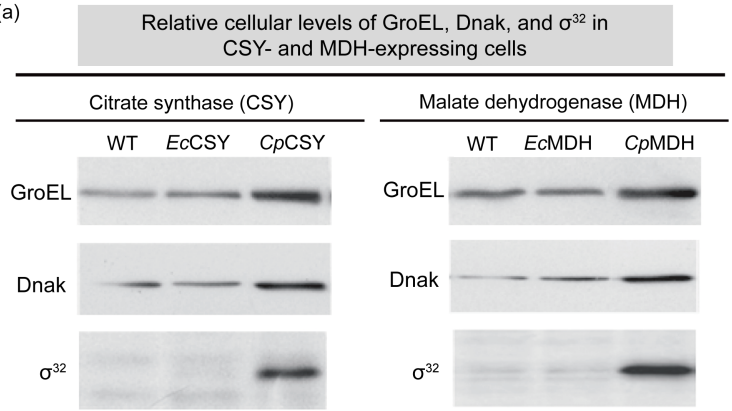
The cellular level of HSPs was determined by Western blotting analysis in heterologously expressing *Cp*CSY, *Cp*MDH, *Ec*CSY and *Ec*MDH and compared to that of cells transformed with empty vector as negative control in strain BL21. *E. coli* cells heterologously expressing *Cp*CSY were grown at 20˚C and accumulation of GroEL and DnaK was found to be significantly higher (**Figure 4(a)**). The densitometric evaluation revealed that the levels of GroEL and DnaK were 3.9-fold and 3.5-fold higher than those of vector control (**Figure 4(b)**), respectively. In contrast, the heterologous expression of *Ec*CSY resulted in a slight increase in levels of HSPs (**Figure 4(a)** and **Figure 4(b)**). The levels of GroEL and DnaK in *E. coli* cells heterologously expressing *Cp*MDH were also higher than those of vector control (BL21) and cells heterologously expressing *Ec*MDH (**Figure 4(b)**).

We further determined the cellular level of σ^{32} in each transformed *E. coli*. As shown in **Figure 4**, the cellular level of σ^{32} increased in *Cp*CSY- and *Cp*MDH-expressing cells (15.9-fold and 22.4-fold respectively). However, the heterologous expression of *Ec*CSY or *Ec*MDH had almost no effect on the relative level of σ^{32}. Overall; these results demonstrated that the heterologous expression of thermolabile proteins, *Cp*CSY and *Cp*MDH, increased the levels of GroEL, DnaK and σ^{32} in *E. coli* cells.

3.3. The Heterologous Expression of *Cp*CSY and *Cp*MDH Enhanced Thermotolerance of *E. coli*

Since heterologous expression of *Cp*CSY and *Cp*MDH increased the cellular level of GroEL, DnaK and σ^{32} in *E. coli*, we examined its effect on their thermotolerance levels. The transformed *E. coli* cells were incubated at

(a)



(b)

Values obtained from western blot using densitometry

	WT	*Ec*CSY	*Cp*CSY		WT	*Ec*MDH	*Cp*MDH
GroEL	1.0	1.5	3.9	GroEL	1.0	0.8	2.7
Dnak	1.0	1.1	3.5	Dnak	1.0	1.5	4.2
σ^{32}	1.0	2.0	15.9	σ^{32}	1.0	1.2	22.4

Figure 4. (a) Levels of GroEL, DnaK and σ^{32} determined by western blot analysis. (b) Numbers indicate levels of GroEL, DnaK and σ^{32} in recombinant *E. coli* relative to those in vector control (wild type, abbreviated as WT) cells. Using ImageJ program, relative levels were calculated by densitometry analysis of the same area from each lane corresponding to migratory position of respective protein.

53˚C for 15 min, and then spotted onto LB agar plates. These plates were grown under permissive conditions and cell viabilities were compared. When the cells were grown on the plate without IPTG in LB medium (no induction of each recombinant protein), and exposed to 53˚C for 15 min, there was almost no growth observed in either of the cells (**Figure 5(a)**). However, in the presence of IPTG in LB medium, *E. coli* cells hetero- logously expressing *Cp*CSY and *Cp*MDH exhibited better survival and quicker growth recovery than cells containing empty vector or cells expressing *Ec*CSY and *Ec*MDH (**Figure 5(a)**).

After 53˚C treatment for 15 min, we further analyzed the growth of each recombinant *E. coli* (CSY-expressing cells grown at 20˚C, and MDH-expressing cells at 30˚C) at regular time interval by measuring optical density values at 600 nm. Growth curve was plotted using optical density values against time interval (**Figure 5(b)**). This data imply that *E. coli* cells expressing *Cp*CSY and *Cp*MDH showed remarkable growth even after high-temperature treatment. On the other hand, cells expressing *Ec*CSY and *Ec*MDH or those containing empty vector

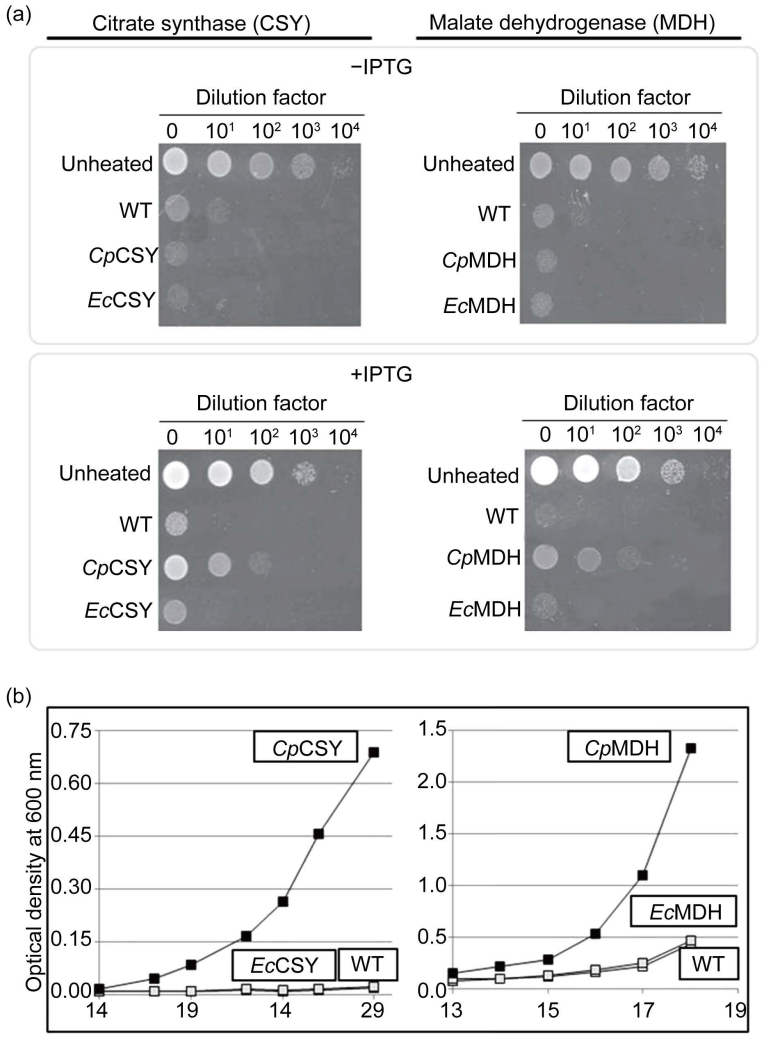


Figure 5. Thermotolerance of recombinant *E. coli*. (a) Dot assay of each recombinant *E. coli* was done for analyzing effect of high temperature on cell survival. Cells were grown, and IPTG was added to induce the expression of CSYs at 20˚C or MDHs at 30˚C for 2 hr. After incubation at 53˚C for 15 min, cells were serially diluted 10-fold with LB, and 2.5 µl of each suspension was spotted onto LB agar plates. These plates were incubated overnight at respective permissive temperatures. (b) Growth curves for each recombinant *E. coli*. Cells expressing each recombinant protein were grown in LB medium till OD_{600} reached to 0.5 and then IPTG was added to induce the protein expression. The 2 hr grown cultures were incubated at 53˚C for 15 min. After that, these cultures were grown at respective permissive temperatures. Cell growth was determined by measuring the absorbance at 600 nm. Symbols: wild type (*triangale*) *Cp*CSY (*filled circle*), *Ec*CSY (*gray circle*), *Cp*MDH (*filled square*), and *Ec*MDH (*gray square*).

showed comparatively lower growth rates. Cell survival and growth rate (without IPTG induction) after high temperature treatment (53˚C) was comparatively lower and almost negligible in diluted fractions (**Figure 5(a)**). These results demonstrated that heterologous expression of thermolabile proteins (*Cp*CSY and *Cp*MDH) helped *E. coli* cells to acquire the enhanced thermotolerance.

4. Discussion and Conclusions

There are numbers of reports about cold-adapted enzymes native to psychrophilic bacteria [27]-[31]. It has also been reported that several enzymes from psychrophilic bacteria exhibit thermolability compared with their mesophilic and thermophilic counterparts [18] [19]. Our *in vitro* analysis using CD measurements and loss of enzymatic activities at comparatively lowers-temperatures demonstrated that the recombinant *Cp*CSY and *Cp*MDH were misfolded at physiological growth temperature of *E. coli* (**Figure 2**). Thus, *Cp*CSY and *Cp*MDH were selected to investigate the enhanced thermotolerance of *E. coli*.

It is well established that accumulation of unfolded or misfolded proteins in a cell is one of the most important factors that triggers heat shock response [4]. Under physiological conditions, the DnaK chaperone system inactivates sigma factor σ^{32} which is asubunit of RNA polymerase specific to the heat shock promoter in *E. coli*. The DnaK interact directly with σ^{32} that mediate its degradation by proteases, such as FtsH [5]-[7] [24]. However, misfolded/unfolded proteins produced under stress conditions compete with σ^{32} for DnaK binding. The σ^{32} released from DnaK becomes available for the expression of HSP genes. This hypothesis has been supported by the observation that the production of structurally unstable firefly luciferase resulted in elevated levels of HSPs in *E. coli* [32]. In this study, we found that the levels of not only GroEL and DnaK but also σ^{32} increased in*E. coli* cells that heterologously expressed *Cp*CSY and *Cp*MDH (**Figure 4**). Given that *Cp*CSY and *Cp*MDH are thermolabile proteins, heterologously expressed *Cp*CSY and *Cp*MDH should be in misfolded/unfolded state even at physiological temperatures, such as 20˚C to 30˚C, in *E. coli*. Thus, it is most likely that the misfolded/unfolded *Cp*CSY and *Cp*MDH sequester the DnaK chaperone system resulting in release of σ^{32}. This situation caused the induced expression of GroEL and DnaK simultaneously. Taken together, our results clearly demonstrate that the heterologous expression of thermolabile proteins allows the heat shock response to be induced at physiological-growth temperature.

HSP families play important roles for cellular protection against various environmental stresses, such as high-temperature, salt, desiccation, organic solvent and oxidative stresses [20] [22] [23] [33] [34]. Present study showed that the *E. coli* cells heterologously expressing *Cp*CSY and *Cp*MDH acquired enhanced thermotolerance (**Figure 5**). This suggests that induction of HSPs by thermolabile proteins contributes to the cellular protection not only from not only to the high-temperature; it may also acquire enhanced tolerance for other kinds of stress such as oxidative stress. Therefore, heterologous expression of thermolabile proteins may be a useful approach for increasing tolerance to various environmental stresses.

Induction of heat shock response by misfolded proteins also reported in eukaryotes [35] [36]. Accordingly, our approach may provide a tool for the improvement of stress tolerance not only in *E. coli* but also in yeasts and plants. To date, many reports have described the effects of constitutive overexpression of only one kind of HSP and its effects on stress tolerance [22] [23]. In contrast, approach used in this work allows induction of various HSPs expression simultaneously. Further studies will clarify the effect of multi-induction of HSP production on various environmental stresses. Furthermore, detailed analysis of the expression patterns of HSPs in these transformants will provide important information on the mechanism by which misfolded/unfolded proteins upregulate the stress response.

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