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Research Article

Enhanced Acetate Tolerance and Recombinant Protein Accumulation in *Escherichia coli* by Transgenic Expression of a Heat Shock Protein from Carrot (*Daucus carota* L.)

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Background: In *Escherichia coli* (*E. coli*) culture, acetate accumulates as an undesirable by-product of aerobic fermentation on glucose and inhibits cell growth and recombinant protein production.

Objectives: We examined whether the heterologous expression of a eukaryotic heat shock protein (Hsp) can confer tolerance to acetate in *E. coli*.

Materials and Methods: Transgenic cell lines (TCLs) heterologously expressing a small heat shock protein (sHsp) from carrot (*Daucus carota* L.), DcHsp17.7, were exposed to heat, sodium acetate, and alkaline conditions. The cell growth and cell viability were examined by measuring $O.D._{600}$ and colony-forming units (CFU), respectively. The His-tagged recombinant alcohol dehydrogenase (ADH) gene cloned in a pET11a expression vector was introduced into TCL1 and expressed by isopropyl β-D-1-thiogalactopyranoside treatment. After purifying using Ni-NTA affinity chromatography, its accumulation levels were examined using SDS-PAGE in the presence of acetate.

Results: TCLs constitutively expressing DcHsp17.7 showed improved growth, cell density, and cell viability under the stress conditions of heat, acetate, and alkaline compared to an empty vector control line. In acetate stress conditions, TCL1 accumulated more cellular proteins (approximately 130%) than the control. The recombinant ADH accumulated to a higher level in TCL1 (2.2-fold at 16 °C) than the control. The addition of acetate reduced the recombinant ADH level by 70% in the control when compared with the absence of acetate. In contrast, recombinant ADH accumulation was not affected by acetate in TCL1. In the presence of acetate, TCL1 accumulated 6.4-fold more recombinant ADH than did the control. Furthermore, recombinant ADH produced in TCL1 showed 1.5-fold higher enzyme activity than that produced in the control in the presence or absence of acetate.

Conclusion: Our study showed that heterologously expressed DcHsp17.7 from carrot can alleviate the negative effects of acetate on *E. coli*.

Keywords: Alcohol dehydrogenase, Alkaline tolerance, Enzyme activity, Heat tolerance, Molecular chaperone

1. Background

Escherichia coli (*E. coli*) is one of the most widely used expression platforms to produce recombinant proteins and secondary metabolites (1). However, some adverse changes that inhibit cell growth and overall metabolism can occur during cultivation. Acetate and other small organic acids frequently accumulate during aerobic fermentation on glycolytic substrates, such as glucose (2). The rapid uptake of glucose in the accelerated cell culture causes the concomitant diversion of acetyl-CoA in the tricarboxylic acid (TCA) cycle to acetate (3). The resulting accumulation of acetate acidifies the growth

medium and disturbs essential cellular processes such as the replication, transcription, and translation of genes by denaturing the structures of DNA, RNA, and proteins (4). The accumulated acetate also directly inhibits the methionine synthesis pathway, leading to the accumulation of homocysteine, a toxic intermediate (5). Accordingly, abundant acetate inhibits overall cell growth, biomass accumulation, and recombinant protein production in *E. coli*.

To relieve the toxic effect of acetate, systematic efforts have been made using genetic engineering techniques. The genes involved in the acetate production pathway were deleted or mutated, or the expression level of related genes was changed to reduce acetate levels (3). For example, the genes for acetate kinase-acetylphosphotransferase (ackA-pta) and pyruvate oxidase (poxB), which are involved in acetate synthesis, were mutated, and phosphoenolpyruvate carboxylase (ppc), which provides oxaloacetate in the TCA cycle, was constitutively overexpressed in different cell lines. In the ackA-pta/poxB knockout and ppc overexpression cell lines, acetate levels decreased, but other unfavorable by-products, lactate and succinate, accumulated, respectively. Acetate metabolism and the TCA cycle are closely related in the production of cellular energy. Thus, the modification of related gene(s) can result in an imbalance of the overall metabolic network and the subsequent inhibition of cell growth. The cAMP receptor protein gene (crp) that regulates genes involved in the TCA cycle was mutated via error-prone polymerase chain reaction (PCR) (6). Although mutant strains showed enhanced acetate tolerance, the modified expression of crp, a master regulatory protein in *E. coli*, resulted in the disrupted expression of more than 400 crp-related genes in the presence or absence of acetate. As such, further studies are required to improve the cell growth and productivity of *E. coli* in the presence of acetate.

2. Objectives

In this study, we examined whether the heterologous expression of a eukaryotic heat-shock protein (Hsp) can confer tolerance to acetate stress in *E. coli*. One of the most fundamental changes that living cells experience in stress conditions is protein denaturation and degradation. Hsps are molecular chaperones that interact with various unfolded proteins to prevent further aggregation and/or correct protein folding, resulting in improved cell survival and growth. Among all organisms, plants have the largest number of Hsps (7). *Escherichia coli* has two small heat-shock proteins (sHsps): IbpA and IbpB (approximately 16 kDa). Conversely, poplar (*Populus trichocarpa*) has 36 different ones. Plant Hsps are localized in different subcellular compartments, such as the cytoplasm, nucleus, mitochondria, endoplasmic reticulum, and peroxisome. Some Hsps are found in specific tissues and organs, such as pollen and seeds, in the absence of stress. These findings suggest that various plant Hsps may have different functional characteristics from their bacterial counterparts. The heterologous expression of eukaryotic Hsps may be able to compensate for the insufficient bacterial Hsp system and provide enhanced stress tolerance and recombinant protein production. We introduced a sHsp gene from carrot (*Daucus carota* L.), *DcHsp17.7* (NCBI accession no. X53851), to *E. coli* BL21 (DE3) and examined cell growth, viability, and protein production in the presence of acetate.

3. Materials and Methods

3.1. Genomic Insertion and Heterologous Expression of DcHsp17.7 *Gene in Transgenic E. coli*

TCLs heterologously expressing DcHsp17.7 were generated using Red/ET-based homologous recombination (Gene Bridges, GmbH, Heidelberg, Germany) (8). Briefly, to generate recombinant DNA constructs, the DNA of the promoter region of lipoprotein (Lpp) (NCBI accession no. NC_000913.2), the *DcHsp17.7* genes from carrot (NCBI accession no. X53851), and Flippase recombinase target (Frt) cassette for homologous recombination were independently amplified from the *E. coli* BL21 (DE3) genomic DNA, the carrot genomic DNA, and the Frt cassette provided by the manufacturer, respectively, using PCR and connected by overlap extension PCR. The forward primer for the Lpp gene promoter and reverse primer for the Frt cassette were designed to contain the sequences of the genomic insertion site, the *yddE* pseudogene (NCBI accession no. NC_012971.2) of *E. coli* BL21 (DE3). The resulting DNA construct, Lpp promoter: *DcHsp17.7* genes: Frt cassette flanked by insertion site sequences, was inserted in the middle of the *yddE* pseudogene of *E. coli* BL21 (DE3) via Red/ET-mediated homologous recombination. Individual TCLs were propagated from independent colonies that survived on kanamycin-containing medium.

The insertion of the *DcHsp17.7* genes in the *E.coli* genome was confirmed by PCR. The genomic DNA was extracted from TCLs and the control and the *DcHsp17.7* genes was amplified using *DcHsp17.7* gene specific primers (Forward:5'-GGGGGGCATATGTCGATCATTCCAAGC-3',Reverse:5'-GGGGGGGCTAGCTTAACCAGAAATATCAATGG-3'; The start and stop codons for *DcHsp17.7* genes were underlined.). The heterologous expression of DcHsp17.7 in TCLs was confirmed via immunoblot analysis using anti-DcHsp17.7 polyclonal antibodies. TCLs were cultured overnight, diluted to $O.D._{600}$= 0.01 with fresh LB medium, and continuously grown with shaking at 37 °C until $O.D._{600}$= 0.6. After centrifuging at 1,360 x *g* at 4 °C for 20 min, the resulting cell pellet was resuspended in protein extraction buffer (25 mM Tris-HCL, pH 7.5, 300 mM NaCl, and 3 mM β-mercaptoethanol) and ultrasonicated (420 W and 20 kHz for a total of 4 min and 40 s with repeated bursts of 10 s of ultrasonication and 30 s of pauses), followed by another round of centrifugation. The proteins in the supernatant were quantified using Bradford assay (9). Equal amounts of proteins (25 µg) were separated using SDS-PAGE (17%), followed by immunoblot analysis (Amersham ECL Prime Western Blotting, GE Healthcare Life Sciences, UK) using anti-DcHsp17.7 polyclonal antibodies. Chemiluminescent signals were captured using the ChemiDoc MP Imaging System (Bio-Rad, Hercules, USA).

3.2. Cell Growth and Viability under Stress Conditions

TCLs and an empty vector control (*E. coli* BL21 containing only Red/ET plasmid) were cultured overnight (37 °C in LB broth containing 15 µg. mL^{-1} kanamycin), diluted to $O.D._{600}$= 0.01 with fresh LB medium (approximately 40 mL in 125-mL flasks), and continuously cultured with shaking for 9 h under various stress conditions. The cells were exposed to 42~48 °C for heat stress. Sodium acetate was added to the culture medium to a final concentration of 50~120 mM for acetate stress. The pH of the culture medium was raised to pH 7.5~9 for alkaline stress. Sodium acetate was added to the medium at a final concentration of 150 or 200 mM with a pH increase to 7.5 for an acetate–alkaline combined stress. $O.D._{600}$ was measured at 1-h intervals.

To examine cell viability, the colony-forming unit was examined. TCL1 and the empty vector control were cultured overnight, diluted, and continuously cultured until $O.D._{600}$= 0.6, as stated above. For stress treatments, culture temperature was raised to 48~50 °C. Sodium acetate was added to the culture medium at a final concentration of 50~120 mM. The pH of the culture medium was raised to pH 7.5~9. For the acetate–alkaline combined stress treatment, sodium acetate was added to a 150 or 200 mM final concentration, and pH was simultaneously raised to 7.5. The cells were then cultured for 60 min, diluted 1:10^3 with fresh LB medium, spread on solid LB plates containing 15 µg.mL^{-1} kanamycin, and incubated at 37 °C overnight. The surviving colonies were counted the following day.

3.3. Protein Accumulation under Acetate Stress

The proteins of TCL1 and the empty vector control were visualized in SDS-PAGE and quantified in the presence or absence of acetate. The two cell lines were cultured overnight and then diluted to $O.D._{600}$= 0.01 in 60 mL fresh LB medium as stated above. Sodium acetate (final concentration, 100 mM) was added to the medium. The cells were then cultured at 37 °C for 9 h with shaking. At 1-h intervals, the cells were collected and centrifuged (37,800 x *g*, 4 °C, 10 min). The resulting cell pellet was resuspended in 3 mL protein extraction buffer, followed by ultrasonication as stated above. Equal volumes of protein samples (4 µL) were resolved in SDS-PAGE (17%) and visualized using Coomassie Brilliant Blue (Bio-Rad). The extracted proteins were quantified using Bradford assay (9).

3.4. Recombinant ADH Expression in the Presence of Acetate

The alcohol dehydrogenase (ADH) gene (*adh*; NCBI accession no. D90421.1) from thermophilic *Geobacillus stearothermophilus* was cloned into a pET11a expression vector at Nhe1 and BamH1 restriction sites (10). A His-tag was attached to the N-terminal of the recombinant protein (His-ADH) for purification. The recombinant plasmid was introduced into TCL1 and the empty vector control line. The two cell lines were cultured overnight, diluted to $O.D._{600}$= 0.01 with fresh LB medium with or without sodium acetate (final concentration, 100 mM), and continuously cultured. At $O.D._{600}$= 0.6, the cells were treated with isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 and 0.5 mM) and continuously cultured at 37 °C and 16 °C for 4 and 16 h, respectively. The proteins were then

extracted using ultrasonication as stated above, and the recombinant His-ADH was purified using Ni-NTA affinity chromatography (His-Bind Resin, Novagen, USA) according to the manufacturer's protocol. Equal volumes of eluents (20 µL) were resolved in SDS-PAGE (17%) and subsequently stained by Coomassie Brilliant Blue (Bio-Rad).

3.5. Enzyme Assay of Recombinant His-ADH

ADH, a major alcohol-metabolizing enzyme, converts ethanol and NAD^+ to acetaldehyde and NADH, respectively. The recombinant His-ADH was induced by 0.5 mM IPTG treatment at 16 °C for 16 h in TCL1 and the empty vector control line as stated above. After Ni-affinity purification, the recombinant ADH (50 µg) was reacted with ethanol (200 µL) in 0.05 M phosphate buffer (pH 8.0) containing 5 mM of NAD^+ at 25 °C for 30 min (11). The amount of NADH produced was measured using spectrophotometry at 340 nm.

4.Results

4.1. Transgenic E. coli Cell Lines Heterologously Expressing DcHsp17.7

In TCLs, the *DcHsp17.7* genes was inserted in the middle of the *yddE* pseudogene in the *E. coli* genome via homologous recombination (**Supplementary Fig. 1A**). The gene was controlled by the promotor of the bacterial *lpp* gene, which encodes outer membrane Lpp, one of the most abundant proteins in *E. coli* (12). The insertion of the DcHsp17.7 genes in the genome of TCLs and the heterologous expression of DcHsp17.7 in TCLs were confirmed in the absence of stress by PCR using *DcHsp17.7* gene-specific primers and immunoblot analysis using anti-DcHsp17.7 polyclonal antibodies, respectively (**Supplementary Fig. 1B, 1C**).

4.2. TCLs Expressing DcHsp17.7 Showed Enhanced Cell Growth and Viability under Heat, Acetate, and Alkaline Stress Conditions

Independent TCLs were originated from individual colonies that formed on the selection medium containing kanamycin for Frt cassette selection. Four TCLs (TCL1, TCL3, TCL6, and TCL7) were selected based on high cell growth rate under normal conditions (8).

We examined whether the heterologous expression of DcHsp17.7 could confer tolerance to heat stress in *E. coli*. When $O.D._{600}$ was measured, TCLs grew faster to a higher cell density compared with the empty vector control at elevated (42~48 °C) temperatures (**Fig. 1A**). TCL1 grew the fastest to the stationary phase in all the examined temperature ranges, followed by other TCLs, and lastly the control. Heat stress negatively affected the growth of all examined cell lines. However, the growth rates and maximum cell density were higher in TCLs than in the empty vector control. At 42 °C, TCL1 grew approximately 1 and 2 h faster during the log phase and reached 20% and 30% higher cell density at the stationary phase compared with the control, respectively. When temperature was further raised to 47 °C, growth inhibition was observed in the control. TCLs still grew to an $O.D._{600}$ of 1.8, while the control grew to an $O.D._{600}$ of 0.8 at 9 h. At 48 °C, TCL1 reached an $OD._{600}$ of 0.8 at 9 h, while the vector control was not able to grow.

The cell viability of TCL1 was also higher than that of the control at elevated temperatures (**Supplementary Fig. 2A**). Under the heat treatment of 48~50 °C for 1 h, TCL1 showed higher cell viability by approximately 10~20% relative to the control.

TCLs were exposed to acetate to examine changes to stress tolerance. Acetate inhibited the growth of TCLs and the empty vector control in a concentration-dependent manner (**Fig. 1B**). However, TCLs showed higher acetate tolerance than the control. At 50 mM, TCLs grew approximately 2 h faster and reached higher cell density than the control. At 100 and 120 mM acetate, TCL1 noticeably grew the fastest, with $O.D._{600}$ reaching 2.0~2.2 at 9 h, followed by the other TCLs. The growth of the control was significantly inhibited, with the maximum $O.D._{600}$ reaching 0.8~1.0 at 9 h. The cell viability of TCL1 was also higher than that of the control in the presence of acetate (**Supplementary Fig. 2B**). At 50~120 mM for 1 h, TCL1 had a higher cell viability by approximately 10~20% relative to the control. Our results showed that the heterologous expression of DcHsp17.7 can confer tolerance to acetate, a toxic by-product, in *E. coli* culture.

Escherichia coli is a neutrophile that favors neutral pH (13). Alkaline conditions, in addition to acidic conditions, are stressful for the bacterium and cause the SOS (14, 15) and heat shock-like responses (15). We examined whether the heterologous expression of DcHsp17.7 could confer tolerance to alkaline

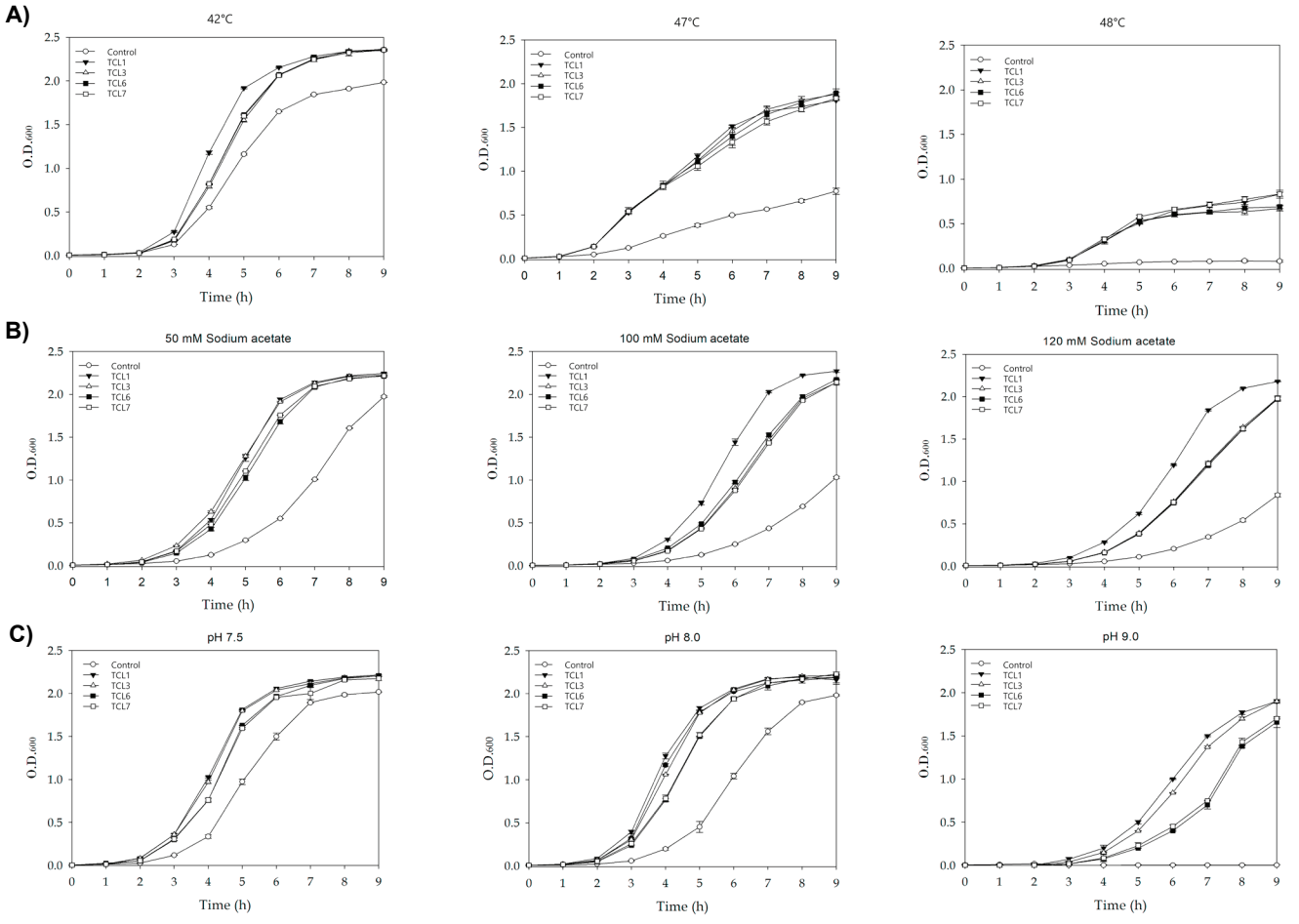

Figure 1. Growth of TCLs under heat, acetate, and alkaline stress conditions. The vector control and four TCLs were cultured at **A)** 42~48°C, **B)** 50~120 mM Sodium acetate, and **C)** pH 7.5~9 for 9 h. The error bars show the standard error of the means.

conditions in *E. coli* (**Fig. 1C**). The empty vector control showed slow growth, reaching the stationary phase approximately 1 h later at pH 8.0 than at pH 7.5. Further increasing pH to 9.0 was even more deleterious to the control, which did not grow for 9 h. In contrast, an increase of pH from 7.5 to 8.0 did not significantly affect the cell growth and maximum cell density in TCLs. At pH 9, the growth of TCLs was slow relative to neutral pH, but they were able to reach $O.D._{600}$ of 1.7~1.9 after 9 h. The cell viability of TCL1 was also higher than that of the control under alkaline conditions (**Supplementary Fig. 2C**). TCL1 showed approximately 5~20% higher cell viability than the control. Our results showed that the heterologous expression of DcHsp17.7 could increase alkaline tolerance in *E. coli*.

4.3. Adverse Effect of Acetate on E. coli Was Alleviated by Raising pH in the Medium

A simple and efficient method of raising pH in the culture medium was suggested to relieve growth inhibition caused by acetate (16). To examine whether acetate-induced growth retardation can be alleviated by raising pH, the bacterial cells were exposed to acetate–alkaline combined stress conditions. In the presence of high levels of acetate (150~200 mM), TCL1 showed the highest growth rate and cell density, reaching $O.D._{600}$ of 2.1 and 2.0 at 9 h, while the control showed an $O.D._{600}$ of only 0.5 and 0.3, respectively (**Fig. 2**).

Increasing pH from 7.0 to 7.5 significantly enhanced cell growth rate in the presence of acetate. At 150 mM acetate, TCL1 grew approximately 1 h faster compared with its growth rate at pH 7.0 (**Fig. 2A**). Growth

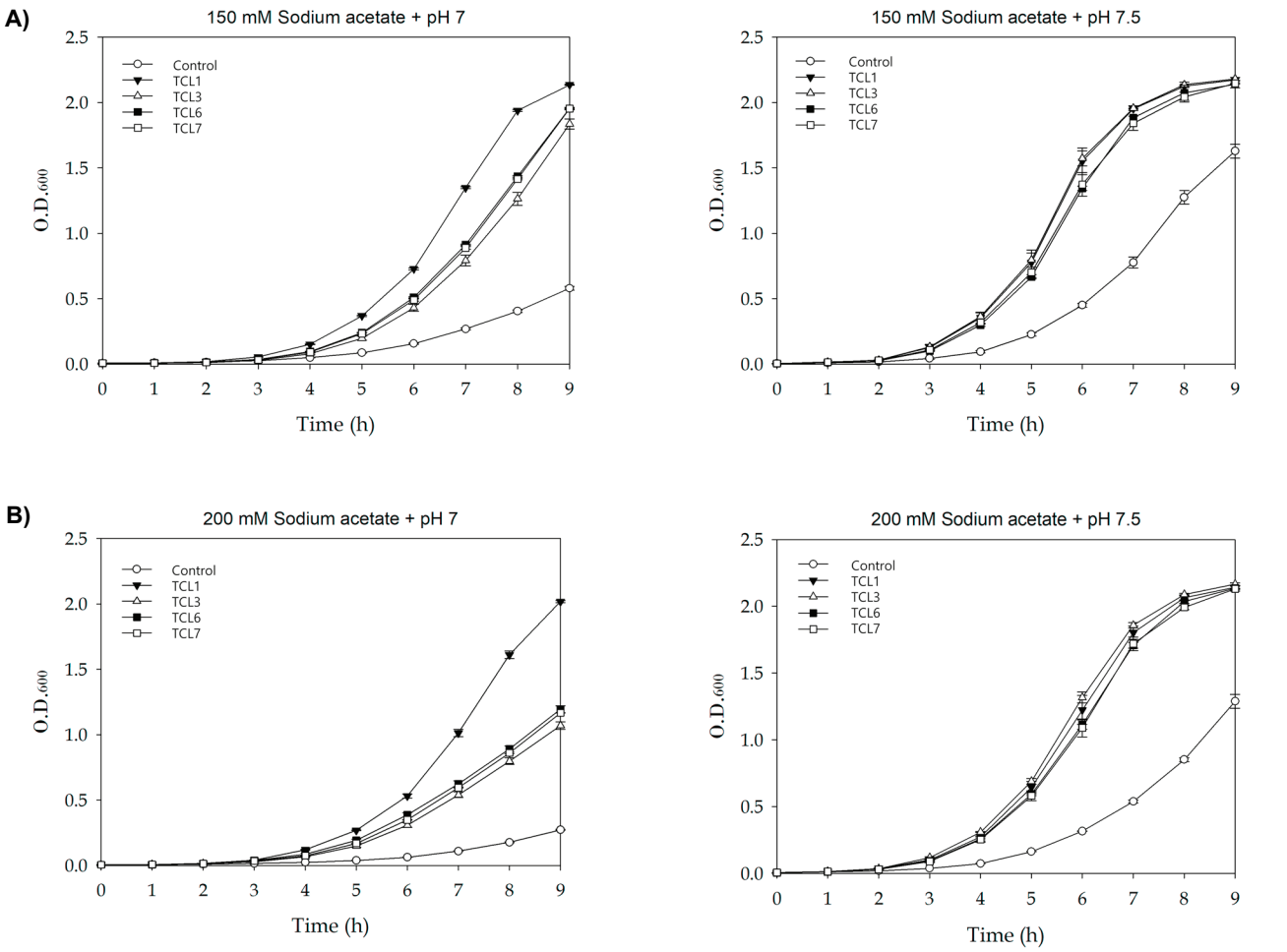

Figure 2. Growth of TCLs under acetate-alkaline combined stress conditions. The vector control and TCLs were cultured in the presence of **A)** 150 mM sodium acetate at pH 7 or pH 7.5 and **B)** 200 mM sodium acetate at pH 7 or pH 7.5 for 9 h.

improvement was more obvious in the control cell line. Increasing pH from 7.0 to 7.5 raised $O.D._{600}$ from 0.5 to 1.5 in the control at 9 h. At 200 mM acetate, TCL1 grew approximately 1.5 h faster at pH 7.5 compared with its growth rate at pH 7.0 (**Fig. 2B**). In the control cell line, cell density was raised from an $O.D._{600}$ of 0.3 to 1.3 at 9 h by raising pH from 7.0 to 7.5. The cell viability of TCL1 was higher by 10~20% relative to the control under the acetate–alkaline combined conditions (**Supplementary Fig. 2D**). Our results showed that the slight increase in pH can further promote the cell growth of TCLs and the empty vector control in the presence of acetate.

4.4. TCL1 Expressing DcHsp17.7 Showed Increased Protein Abundance under Normal and Acetate Conditions

Hasps are molecular chaperones that can promote protein production, folding, and stability (7). We examined whether the heterologous expression of DcHsp17.7 can enhance protein accumulation in *E. coli* in the presence of acetate. The overall protein band profile in SDS-PAGE was similar in TCL1 and the control (**Fig. 3A-3D**). However, TCL1 showed enhanced protein accumulation in the presence or absence of acetate. Under normal growth conditions, TCL1 produced cellular protein at a faster rate than the control (**Fig. 3E**). During the log phage, TCL1 showed a 20~30% greater protein abundance relative to the control. The protein levels in TCL1 reached saturation at 6 h, but those in the control gradually increased until 9 h.

Acetate significantly inhibited protein accumulation in both cell lines (**Fig. 3C, 3D**). However, TCL1 accumulated more cellular protein than the control in

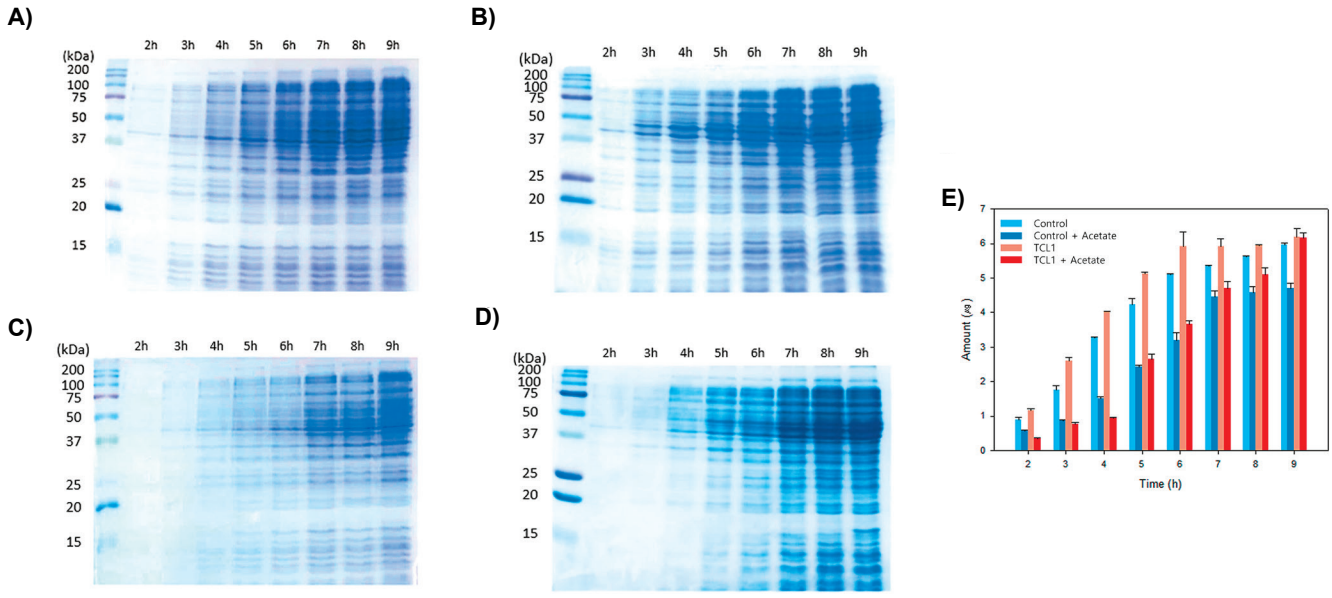

Figure 3. Protein accumulation in TCL1 in the presence of acetate. **A, C)** The control and **B, D)** TCL1 were cultured under **A, B)** normal and **C, D)** sodium acetate (100 mM) conditions for 9 h. Proteins were extracted at 1-h intervals, subjected to SDS-PAGE (17%), or **E)** quantified using Bradford assay to compare protein abundance.

the presence of acetate. TCL1 continuously increased protein abundance in the presence of acetate and produced a similar amount of cellular protein to unstressed TCL1 at 9 h (**Fig. 3E**). In contrast, the control cell line could not overcome the acetate-induced inhibition. At 9 h, protein abundance in the acetate-stressed control cell line was approximately 30% lower than in the unstressed control.

4.5. TCL1 Expressing DcHsp17.7 Showed Increased Recombinant ADH Accumulation

We examined whether the heterologous expression of DcHsp17.7 can increase recombinant protein accumulation in *E. coli* in the presence of acetate. The ADH gene from a thermophile, *G. stearothermophilus*, was cloned in a pET11a expression vector and induced by IPTG treatment at either 37 °C or 16 °C, the latter of which allows slow cell growth with continuous recombinant protein induction. At both temperatures, induced ADH protein accumulation (approximately 38 kDa) was confirmed in the cell extracts of TCL1 and the empty vector control by SDS-PAGE (**Fig. 4A, 4D**). The abundance of other bacterial proteins, especially the ones smaller than ADH, was reduced in TCL1. When purified recombinant ADH levels were compared, in both the control and TCL1, the low temperature of 16 °C was more effective at inducing recombinant ADH than the normal temperature of 37 °C (**Fig. 4B, 4E**). TCL1 produced more recombinant ADH than the control. At 37 and 16 °C, TCL1 accumulated four and twofold more recombinant ADH than the control in the absence of acetate, respectively (**Fig. 4C, 4F**). The addition of acetate significantly reduced the abundance of recombinant ADH in the control at both temperatures. Conversely, acetate did not affect recombinant ADH accumulation in TCL1. The accumulation of recombinant ADH was similar in TCL1 regardless of acetate at both temperatures. TCL1 accumulated 6.4-fold more recombinant ADH than the control in the presence of acetate at 16 °C (**Fig. 4F**).

4.6. Heterologous Expression of DcHsp17.7 Increased Recombinant ADH Activity

It is important that the expressed recombinant proteins remain functional after cell lysis and purification. We examined whether the activities of recombinant ADH produced either in the presence or absence of DcHsp17.7 were different. The same amount of recombinant ADH was purified from TCL1 and the control and was used in the enzyme reaction. In normal growth conditions, the

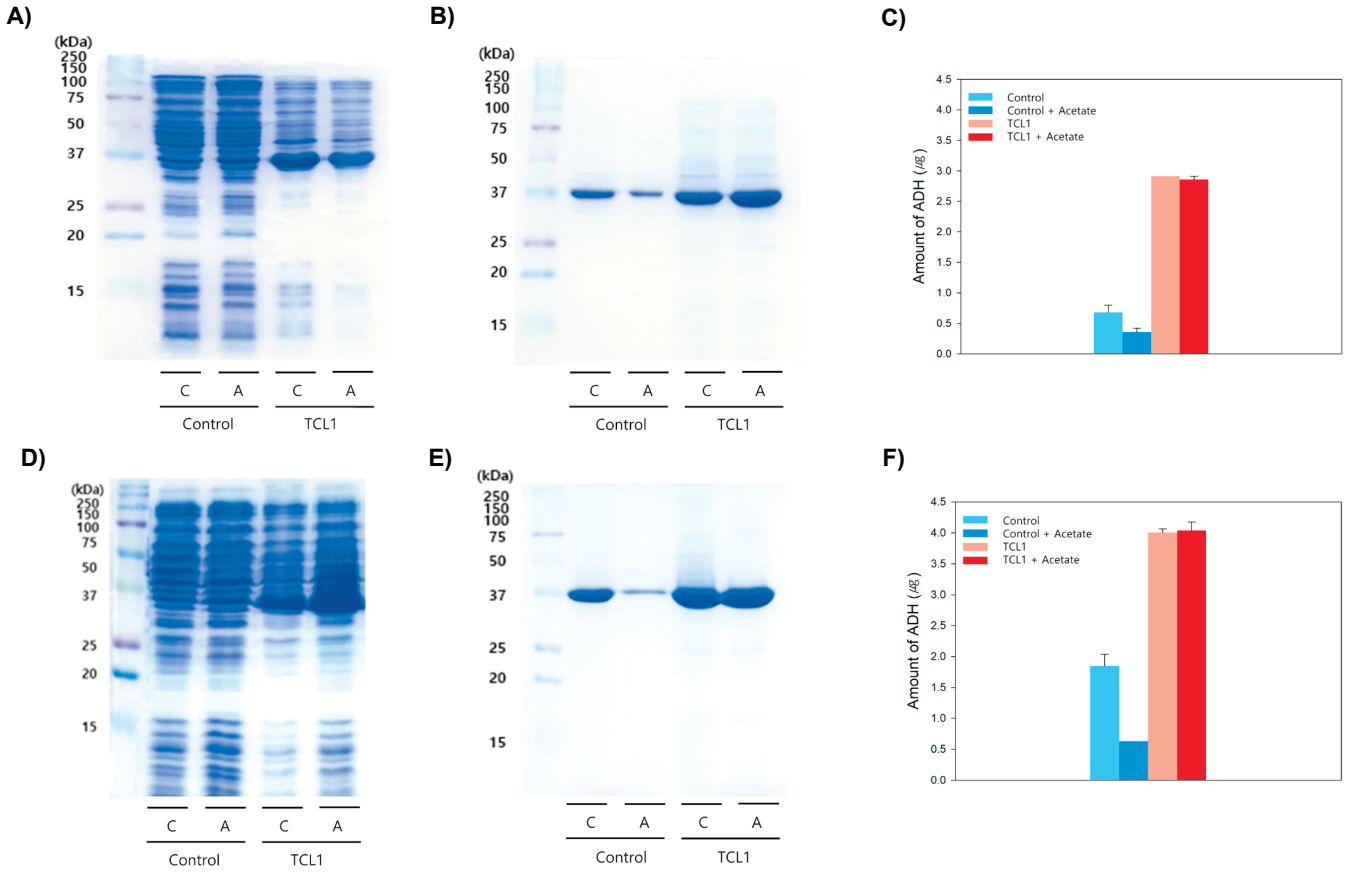

Figure 4. Recombinant ADH accumulation in TCL1 in the presence of acetate. Adh gene was induced by isopropyl β-D-1-thiogalactopyranoside treatment at **A, B, C)** 37 °C and **D, E, F)** 16 °C for 4 and 16 h, respectively. **A, D)** The total proteins and **B, E)** His-tagged recombinant ADH purified using Ni-NTA affinity chromatography were displayed in SDS-PAGE (17%). **C, F)** The purified recombinant ADH was quantified using Bradford assay and graphed to compare the protein levels. The error bars show the standard error of the means. a: cultured in LB medium with 100 mM sodium acetate, c: cultured in LB medium, control: E. coli BL21 containing Red/ET plasmid, TCL1: transgenic *E. coli* BL21 constitutively expressing DcHsp17.7.

recombinant ADH produced in TCL1 was more active than the protein produced in the control (**Fig. 5A**). At 30 min, the recombinant ADH produced in TCL1 was 1.5-fold more active than that produced in the control cells. In the presence of acetate (100 mM), the recombinant ADH produced in both cell lines showed lower enzyme activity by 10%~15% than that produced under normal conditions (**Fig. 5B**). However, the recombinant ADH produced in the acetate-treated TCL1 was 1.6-fold more active than that produced in the acetate-treated control cells. Our results showed that the heterologous expressed DcHsp17.7 can increase not only the yield of recombinant ADH produced but also the activity of the expressed enzyme. It is possible that DcHsp17.7 acts as a molecular chaperone to properly fold and stabilize the recombinant ADH.

5. Discussion

In this study, TCLs heterologously expressing DcHsp17.7 were tolerant to heat, acetate, and alkaline conditions relative to the empty vector control. Considering that these are major stressors in industrial fermentation, stress-tolerant TCLs can be useful for biotechnological application. Previously, transgenic *E. coli* expressing thermotolerant MetA, involved in methionine biosynthesis, showed enhanced tolerance to heat and acetate (17). This and our results suggest that acetate tolerance can be developed by genetic engineering in *E. coli*.

The TCLs examined in this study have advantages over the commonly used plasmid-based transgenic *E. coli*. The *DcHsp17.7* genes was inserted into the bacterial genome, which is stable through repeated cell division.

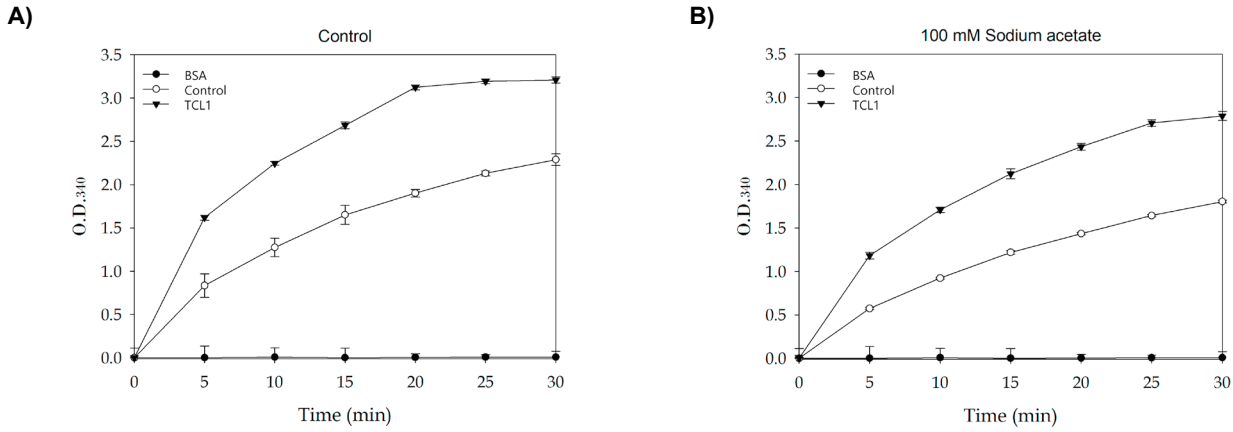

Figure 5. Enzyme activity of the recombinant ADH produced in TCL1. The recombinant ADH (50 µg) purified from TCL1 and the control grown under **A)** normal and **B)** sodium acetate (100 mM) conditions was reacted with ethanol and NAD^+ (5 mM) in 0.05 M phosphate buffer (pH 8.0) at 25 °C. Bovine serum albumin (BSA) was used as a negative control. $O.D._{340}$ was measured to quantify the produced NADH. The error bars show the standard error of the means.

Furthermore, transgene expression was driven by the constitutive *E. coli lpp* gene promoter. Consequently, the chemical or physical induction treatments for recombinant protein expression are not required for these TCLs. Considering the possible toxic effects of chemical inducers, such as the frequently used IPTG (18) or temperature upshifts for heat-inducible induction systems on *E. coli* (1), the usage of an endogenous promoter is advantageous. Bacterial sHsps, IbpA and IbpB, are not present or are present at low levels in unstressed *E. coli*. However, it was reported that the accumulation of aggregation-prone proteins could induce IbpA expression in the absence of stress (19). The constitutively expressed DcHsp17.7 could protect bacterial proteins from aggregation under normal growth conditions.

In research and/or industrial applications, *E. coli* can be exposed to elevated temperatures. Culture temperature is often raised to activate heat-inducible expression systems for recombinant protein production (20, 21). Simultaneous saccharification and fermentation for ethanol production can also require temperature elevation (22). However, at elevated temperatures, cell growth and overall metabolism are inhibited (23). Thermotolerant TCLs expressing DcHsp17.7 can be useful for industrial applications including temperature elevation.

Heat shock genes, such as *GroEL* (Hsp60), *DnaK* (Hsp70), *HtpG* (Hsp90), and *IbpB* (sHsp), are upregulated in *E. coli* at elevated temperature (22). Heat-shock response and acetate accumulation are closely related in *E. coli*. At 47.5 °C, *E. coli* deplete glucose and accumulate high amounts of acetate (24). At 42 °C, a decrease in biomass coincides with increased acetate accumulation in heat-stressed *E. coli* (22). The temperature elevation causes the downregulation of some genes involved in the TCA cycle, which can lead to acetate formation. It is possible that the heterologously expressed DcHsp17.7 may protect some bacterial proteins from acetate-induced damages. The possible direct and/or indirect relations between Hsps and acetate tolerance remain to be elucidated. To our knowledge, this is the first study reporting that the overexpression of a Hsp can confer tolerance to acetate-induced toxicity.

There are only a limited number of studies available on the alkaline stress response in *E. coli*; most pH-related studies are focused on acidic conditions (25). Transcriptome and proteome analyses have reported the differential gene expression and protein abundance in *E. coli* under alkaline stress relative to neutral pH (26); genes involved in respiratory chain complexes, flagella, and chemotaxis were repressed. In contrast, the genes involved in ATP synthesis were upregulated. For Hsp genes, the results were not consistent. In an early study, protein labeling analysis showed that DnaK and GroEL were induced over 6-fold at pH 8.8 compared with pH 7.1, suggesting the possible role of Hsps in alkaline tolerance (15). However, a later study reported that

DnaK and ClpAB (Hsp100) were repressed in alkaline-stressed *E. coli* (27). It was suggested that alkaline-induced toxicity may result from the inhibition of alkaline-sensitive enzymes (25). The increased alkaline tolerance in TCLs may result from the molecular chaperone activity of DcHsp17.7 to protect bacterial proteins, possibly including alkaline-sensitive cellular enzymes and/or proteins, by rendering them functional in the stress condition.

Our results showed that acetate toxicity can be reduced by raising the pH of the culture medium, suggesting the possible changes related in acetate metabolism in *E.* coli grown under moderate alkaline conditions. The levels of intracellular acetate concentrations decrease at pH 7.5, compared with those at pH 6.5, when sodium acetate (final concentration, 50–300 mM) was added to the culture medium (16). The authors suggested that differential cell membrane regulation may be the cause of reduced intracellular acetate under alkaline conditions. At pH 9.0, the expression levels of proteins involved in acetate formation, such as pyruvate dehydrogenase, were decreased, suggesting a possible direct effect of alkaline pH on reduced acetate levels in *E. coli* (27).

Acetate affects gene expression and protein synthesis, particularly for genes involved in transcription, translation, and stress response (28). It inhibits protein synthesis, particularly recombinant protein production (4). It hinders methionine biosynthesis by inhibiting related enzymes in the pathway (5, 17). In TCLs, the levels of proteins were not affected by acetate treatment, suggesting that the heterologously expressed DcHsp17.7 in TCLs may be able to protect enzymes and/or proteins involved in protein synthesis from acetate stress.

Previously, two bacterial sHsps, IbpA and IbpB, were overexpressed in *E. coli* in order to enhance recombinant protein production (29). The resulting transgenic *E. coli* produced more cytosolic green fluorescent protein than control lines. The two Hsps were found in inclusion bodies, suggesting their possible roles in protecting recombinant proteins from degradation. The ATP-independent sHsps mainly function to hold partially denatured proteins, preventing them from further aggregation, and deliver them to Hsp70 for refolding (30). The co-overexpression of a sHsp and Hsp70 would be a reasonable next approach to further improve cell growth and recombinant protein accumulation.

To overcome the adverse effect of acetate on recombinant protein accumulation, previous studies developed transgenic *E. coli* that generated reduce levels of acetate. For example, low levels of acetate (50 mM) significantly inhibit cell growth and recombinant Thermus maltogenic amylase (ThMA) production by 90% (31). Compared with the unmodified control, the double-mutant transgenic *E. coli* with both acetate kinase (*ackA*) and phosphotransacetylase (*pta*) genes mutated and the triple-mutant transgenic *E. coli* with *ackA-pta*-Lactate dehydrogenase (*ldh*) genes mutated produced reduced levels of acetate and a higher yield of recombinant ThMA production. This is different from the present study that showed acetate tolerance with improved cell growth and recombinant protein accumulation in the presence of 100 mM acetate.

6. Conclusion

Our study showed that DcHsp17.7 can improve cell growth and protein accumulation in *E. coli* under normal and stress conditions. Hsps appear to be promising gene candidates to develop useful TCLs with improved stress tolerance and recombinant protein accumulation. Unlike many proteins that only interact with designated partners, Hsps interact with various nonspecific protein substrates to improve their folding, stability, and function. The identification of useful Hsps outside of the bacterial domain can be helpful to develop useful transgenic *E. coli* cell lines for industrial applications.

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