博士論文

Improvement of The Heat Resistance Ability of
Thermotolerant *Zymomonas mobilis* TISTR 548

（耐熱性 *Zymomonas mobilis* TISTR 548 の耐熱性の改良）

2020 年 9 月

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LIST OF ABBREVIATIONS

% w/v	percent weight by volume
^{o}C	degree celcius
$AlCl_3$	alumunium chloride
ATP	adenosine triphosphate
bp	base pair
$CoCl_2$	cobalt (II) chloride
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
et al.	et alii (Latin), and other
$FeCl_3$	ferric chloride, iron (III) chloride
g	gram
g/L	gram per liter
GSH	glutathione
h	hour (s)
H_2O_2	hydrogen peroxide
i.e.	ed est (Latin), that is
KCl	potassium chloride
$MgCl_2$	Magnesium chloride
min	minute (s)
mL	mililiter
mM	milimolar
$MnCl_2$	manganese (II) chloride
NaCl	Sodium chloride
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form
NaH_2PO_4	monosodium phospate
Na_2HPO_4	sodium phosphate dibasic
$NiCl_2$	nickel (II) chloride
nm	nanometer
OD	optical density
OD_{550}	optical density at 550 nm wavelength

pdc	pyruvate decarboxylase
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
rpm	revolutions per minute
s	second
SD	standard deviation
YPD	yeast-peptone-glucose medium
µg/µL	microgram per microliter
µg.ml^{-1}	microgram per mililiter
µL	microliter
µM	micromolar
µm	micrometer
$ZnCl_2$	zinc chloride

CHAPTER 1

GENERAL INTRODUCTION

World energy consumption is expanding with the increase in the world's population and economic development. However, the available amount of fossil fuel is not sufficient to meet the energy demand in the future (Harun *et al*., 2010; Sivakumar *et al*., 2010). Moreover, the use of fossil fuels has negative environmental consequences such as the greenhouse effect, which in turn is causing global warming and climate change (Ho *et al*., 2013). Thus, the depletion in the supply of fossil fuels is driving efforts to find alternative energy sources that are both economically profitable and environmentally safe (Mussato *et al*., 2010; Ho *et al*., 2013). Biofuel is defined as a renewable energy generated from biological materials which is friendly to the environment. The carbon balance of biofuel is almost neutral when compared with the carbon balances of petroleum-derived fuels. The two most common types of biofuel in use today are bioethanol and biodiesel. In the early 2000s, the much worldwide attention was given to bioethanol production. Several countries including the USA, Brazil, China, and Canada are now using bioethanol as an alternative energy source. Bioethanol is an alcohol that is produced by fermentation and it can be used as a fuel for vehicles in its pure form. However, it is usually used as a gasoline additive to increase octane and improve vehicle emissions (Shalaby, 2013).

Bioethanol is classified into three types (Jambo *et al.*, 2016) according to its sources. First-generation bioethanol is generally produced from the fermentation of sugars. Consequently, it is often produced by using edible biomass like sugarcane, corn, cereal crops, potato, cassava, and sugar beets, and it production therefore competes with the food industry. Second-generation bioethanol and third-generation bioethanol are produced by using non-

edible lignocellulosic biomass and algal biomass, respectively, as sources of feedstock, and their production therefore appears to be more sustainable. Bioethanol fermentation is generally performed at temperatures in the range of 25-35˚C according to the optimal growth temperatures of ethanologenic microorganisms that are classified into mesophiles (Samappito *et al.,* 2018). It has been confirmed that there is almost no growth or production of ethanol by ethanologenic microbes at temperatures higher than 35˚C (Aldiguier *et al*., 2004). Bioethanol fermentation itself is exothermic and much heat is generated in the process. Coupled with the problem of global warming, temperature control, particularly that in large-scale fermentation, has becomes challenging (Samappito *et al.,* 2018). Hence, temperature control at a suitable temperature is necessary for the growth of microorganisms used for fermentation.

The application of high-temperature ethanol fermentation (HTEF) for bioethanol production has many benefits since it enables reduction in cooling expenses and contamination risk (Zhang *et al*., 2015). Other benefits are related to a high fermentation rate, facilitation of product recovery, and more efficient simultaneous saccharification and fermentation (SSF). However, the generation of heat during the fermentation process inhibits the growth of cells and thus reduces cell viability and reduces the yield of ethanol (Thanonkeo *et al*., 2007a; Sootsuwan *et al*., 2013, Techaparin *et al*., 2017). Therefore, thermotolerant ethanologenic microbes are required for stable HTEF.

Zymomonas mobilis, a facultative anaerobic Gram-negative bacterium, proficiently ferments sugars to ethanol by using the Entner-Doudoroff (ED), glyceraldehyde-3-phosphate to pyruvate (GP), and pyruvate to ethanol (PE) pathways (Ley *at al.,* 1977; Tribe *et al*., 1980). Since this organism lacks 6 phosphofructokinase and also 2-oxoglutarate dehydrogenase and malate dehydrogenase, which are critical enzymes for the Embden-Meyerhof-Parnas (EMP) pathway and TCA cycle (Sprenger *et al.,* 1996; Seo *et al.,* 2005), the ED-GP-PE pathway helps this bacterium to maintain the level of glucose flux by

providing sufficient ATP due to expression of many related enzymes. As a result, biomass formation is less and ethanol production is more efficient compared to those for *Saccharomyces cerevisiae* (Rogers *et* al., 1980; Thanonkeo *et al.,* 2005; Matsushita *et al*., 2015). Moreover, the ethanol yield from *Z. mobilis* is 5–10% higher than that from *S. cerevisiae*. The maximum amount of ethanol produced by *Z. mobilis* can reach approximately 97% of the theoretical yield, whereas *S. cerevisiae* can produce only 90–93% of the theoretical yield (Hayashi et al. 2012). Additionally, *Z. mobilis* has a high tolerance to high levels of ethanol and high sugar concentrations (Sprenger, 1996; Swings and De Ley, 1977; Sootsuwan *et* al., 2013; Charoensuk *et al*., 2017, Carreon-Rodriguez *et al*., 2019).

Z. mobilis is a mesophilic bacterium like *S. cerevisiae* that generally grows at temperatures around 30$_{o}$C. However, some strains are relatively thermotolerant. TISTR 548, a strain isolated from Thailand, showed high rate of growth and ethanol production at high temperatures under static and shaking conditions (Sootsuwan *et* al., 2007; Thanonkeo *et al.*, 2011; Samappito *et al.,* 2018). By using the 2-times cultivation method, it was confirmed that the critical high temperature of this strain is 38$_{o}$C (Kosaka *et al.,* 2019). Besides the results for growth and ethanol production, this CHT result clearly indicated that TISTR 548 is more thermotolerant than CP4 (Matsushita *et al.,* 2015; Kosaka *et al.,* 2019). Several studies were carried out to improve the capabilities of TISTR 548 in order to cope with heat stress. Stepwise thermal adaptation was performed and resulted in mutants 200M and MAS1 that have CHTs at 2$_{o}$C and 3$_{o}$C higher, respectively, than that of TISTR 548 (Kosaka *et al.,* 2019). In a study performed by Samappito *et al*. (2018), ZM AD41, another thermo-adapted strain is able to maintain growth at 41$_{o}$C, was obtained. Those results suggested that accumulation of mutations may allow the discovery of new thermotolerant strains, in turn suggesting the possible role of innate genomic factors in thermal adaptation (Kosaka *et al.,* 2019). Hold on

this understanding, a genetical investigation may be beneficial for conversion of non-thermotolerant to thermotolerant microbes.

As a result of heat exposure, microbial cells will produce stress proteins as a part of the stress response. Some heat shock proteins (HSPs) were visibly recognized when TISTR 548 cells were exposure to a temperature of 40$_{o}$C (Thanonkeo *et al*., 2007a). Intracellular oxidative stress also accumulates during heat exposure (Noor *et al*., 2009, Murata *et al*., 2018) and can induce cell death (Davidson *et al*., 1996). The expression of ROS-scavenging enzymes (RSEs) such as catalase or superoxide dismutase contributes to the degradation of intracellular ROS, which results in an increase in thermotolerance (Davidson *et al*., 1996; Noor *et al*., 2009; Basak and Jiang, 2012; Nantapong *et al*., 2019). Since ROS accumulate in cells of *Z. mobilis* strains at their CHTs (Kosaka et al., 2019), macromolecules like proteins are possibly exposed to ROS. Consequently, various type of protein damage such as unfolding, misfolding, aggregation, or denaturation may induce functional changes and disturb cellular metabolism (Cabiscol *et al*., 2000). Therefore, many HSPs are molecular chaperones (Tomoyasu *et al*., 1998; Kitagawa *et al.*, 2002; Zolkiewski *et al*., 2012 Mogk *et al.*, 2015) and proteases (Jones *et al.*, 2002), components that may play an important role at high temperatures to protect cells against protein damage by repairment or degradation.

On the other hand, results of genome-wide analysis of the genes required for survival at CHTs among mesophilic bacteria groups represented by *Escherichia coli*, *Acetobacter tropicalis*, and *Zymomonas mobilis* showed that the functional classes of thermotolerant genes were categorized as metabolism, membrane stabilization, transporter, DNA repair, tRNA modification, protein quality control, translation control, cell division, and transcriptional regulation (Soemphol *et al*., 2011; Charoensuk *et al.*, 2017; Murata *et al*., 2018). However, there are only a few genes for reactive oxygen species (ROS)-scavenging enzymes (RSEs) and heat shock proteins (HSPs) as thermotolerant genes in those mesophilic

bacteria (Soemphol *et al*., 2011; Charoensuk *et al.*, 2017; Murata *et al*., 2018). In this study, to understand the contribution of genes for RSEs and HSPs to thermotolerance, the effects of enhanced expression of these genes on survival at the critical high temperature (CHT) in *Z. mobilis* TISTR 548 were examined.

Heat stress does not only inhibit the growth and fermentation ability of *Z. mobilis* cells (Thanonkeo *et al*., 2007a) but also alters the fluidity of the plasma membrane (Carey and Ingram, 1983; Moreau *et al*., 1997). Like ethanol stress, excessive heat probably disrupts cellular ionic homeostasis, reduces metabolic activity and leads to cell death. The presence of cations in metabolic processes and physiological functions is crucial for HTEF by ethanologenic microorganisms. Metal ions have been shown to improve growth at a high temperature for various microorganisms including *S. cerevisiae*, *Pseudomonas aeruginosa*, *E. coli., Lactobacillus* strains and even *Z. mobilis* (Thanonkeo *et* al., 2007a; Reid *et al*., 2009; Huang and Chen, 2013; Lam *et al.,* 2014; Charoensuk *et al*., 2019), but the maximum limit of concentration must be considered since a high level of metal ions may be toxic to some cells (Gadd, 1992). It was also suggested that the concentration of metal ions in the fermentation medium affects the productivity for HTEF. In *Z. mobilis*, investigation of metal ions other than Mg^{2+} (Thanonkeo *et al*., 2007a) has not been performed yet. In this study, we examined the effects of various metal ions including Mn^{2+}, Ni^{2+}, Co^{2+}, Al^{3+}, Fe^{3+}, Zn^{2+}, Mg^{2+}, K^{+}, and Ca^{2+}, on growth of *Z. mobilis* TISTR 548 at its CHT. The mechanisms effect of some metal ions on cell physiology at the CHT would be elucidated. The effects of Mg^{2+} and K^{+} were also investigated in thermosensitive mutants of *Z. mobilis* TISTR 548 were also investigated.

CHAPTER 2

Improvement of thermotolerance of *Zymomonas mobilis* by genes for reactive oxygen species-scavenging enzymes and heat shock proteins

2.1 Abstract

Thermotolerant genes, which are essential for survival at a high temperature, have been identified in three mesophilic microbes, including *Zymomonas mobilis*. Contrary to expectation, they include only a few genes for reactive oxygen species (ROS)-scavenging enzymes and heat shock proteins, which are assumed to play key roles at a critical high temperature (CHT) as an upper limit of survival. We thus examined the effects of increased expression of these genes on the cell growth of *Z. mobilis* strains at its CHT. When overexpressed, most of the genes increased the CHT by about one degree, and some of them enhanced tolerance against acetic acid. These findings suggest that ROS-damaged molecules or unfolded proteins that prevent cell growth are accumulated in cells at the CHT.

2.2 Introduction

Microorganisms intrinsically have an upper temperature limit for survival called a critical high temperature (CHT) (Matsushita *et al*., 2015; Kosaka *et al*., 2019). Genome-wide analysis of three mesophiles, *Escherichia coli*, *Acetobacter tropicalis*, and *Zymomonas mobilis*, by screening thermosensitive mutants either with a single-knockout mutant library or with a transposon- inserted mutant library has revealed that about 1.5% of genomic genes, called thermotolerant genes, are responsible for cell survival at a CHT (Charoensuk *et al*., 2017; Murata *et al*., 2018), but there is no sufficient information to conclude that a mesophile with a larger number of genomic genes has a larger number of thermotolerant genes and thus

tends to be more temperature-resistant. Other factors including gene expression of key proteins may also contribute to the degree of thermotolerance. Thermotolerant genes are categorized into nine groups, including genes for metabolism, membrane stabilization, transporter, DNA repair, tRNA modification, protein quality control, translation control, cell division, and transcriptional regulation (Murata *et al*., 2011, 2018; Soemphol *et al*., 2011; Charoensuk *et al*., 2017), which are mostly related to fundamental activities of cells. The CHT differs to some extent from strain to strain even in the same species such that relatively thermotolerant strains can be isolated in tropical areas, assuming that thermotolerant strains have adapted to the environmental temperature. The thermotolerance of mesophiles, *E. coli* W3110, *Z. mobilis* CP4, and *Z. mobilis* TISTR 548, has been improved by *in vivo* thermal adaptation, suggesting that they have a genomic capacity for adaptation to higher temperature environments (Kosaka *et al*., 2019). The capacity, however, is limited to 2–3°C, and the variation of thermal adaptation is also restricted. The change of only a few degrees is physiologically important, which was determined by an accurate method, called a two-step cultivation assay that eliminates the effects of start temperature and can distinguish CHT differences between two different strains of the same species (Kosaka *et al*., 2019).

The thermotolerance of microbes is remarkably beneficial for stable fermentation. Ethanologenic microbes, for example, are exposed to heat stress in the ethanol fermentation process (Attfield, 1997; Wang *et al*., 2007) due to its exothermic reaction (van Uden and da Craz Duarte, 1981; Ghose and Bandyopadhyay, 1982). Heat stress has a negative impact on their growth or viability (Basso *et al*., 2008; Babiker *et al*., 2010) and prevents their fermentation ability. These negative effects are enhanced in the presence of other factors, including a low pH, a high ethanol concentration, or a high osmolarity (Piper, 1995; Carmelo *et al*., 1998; Ciani *et al*., 2006; Coleman *et al*., 2007; Gibson *et al*., 2007; Pizarro *et al*.,

2007). Thus, heat stress should be avoided for stable and effective fermentation by chilling fermentation reactors. Thermotolerant microbes enable high- temperature fermentation (HTF) to be performed, thus reducing the costs for chilling reactors in the bioconversion process of biomass to fuels or chemicals and for biomass hydrolysis in simultaneous saccharification fermentation and thus preventing contamination of other microbes (Murata *et al*., 2015).

A higher temperature results in accumulation of more oxidative stress in *E. coli* (Noor *et al*., 2009), and oxidative stress is involved in heat-induced cell death as has been shown for *Saccharomyces cerevisiae* (Davidson *et al*., 1996), being consistent with findings that overexpression of genes for catalase and superoxide dismutase is able to increase the degree of thermotolerance (Nantapong *et al*., 2019) and that the thermotolerance increases under anaerobic conditions (Davidson *et al*., 1996; Davidson and Schiestl, 2001). It is thus assumed that the impact of the CHT causes intracellular oxidative stress to elicit harmful effects on cells as a secondary stress. However, only one gene and no gene for reactive oxygen species (ROS)- scavenging enzymes (RSEs) was found as a thermotolerant gene in *A. tropicalis* and *Z. mobilis*, respectively (Soemphol *et al*., 2011; Charoensuk *et al*., 2017). The CHT would also cause damage of proteins to be unfolded or denatured. Surprisingly, no genes for general heat shock proteins (HSPs), except for *degP*, *dnaK*, and *dnaJ* in *E. coli* (Murata *et al*., 2011, 2018), *degP* in *A. tropicalis* (Soemphol *et al*., 2011), and *degP* in *Z. mobilis* (Charoensuk *et al*., 2017), have been identified as thermotolerant genes.

In this study, we thus examined the effects of increased expression of genes for RSEs and HSPs on cell survival at the CHT in *Z. mobilis*. Increased expression of most of the genes tested raised the CHT and reduced ROS compared to the controls. These findings together with previous findings suggest that the CHT is determined by functional

contributions of several factors that prevent the accumulation of damaged macromolecules in cells in addition to fundamental activities by thermotolerant genes identified previously.

2.3 Materials and methods

2.3.1 Materials

Oligonucleotide primers were purchased from Greiner Bio-One (Japan). A DNA purification kit, gel extraction kit, and one- step RT-PCR kit were from Qiagen (Japan). Restriction enzymes were from Biolabs (Japan) and Takara (Japan). PrimeSTAR DNA polymerase and an In-Fusion HD cloning kit were purchased from Takara (Japan). *E. coli* DH5α was from Toyobo (Japan). Yeast extract, peptone, tryptone, and agar were from Nacalai Tesque (Japan). Glucose and NaCl were from Sigma-Aldrich (United States). Chloramphenicol was from Boehringer Mannheim GmbH (Germany). Other chemicals used in this study were of analytical grade.

2.3.2 Bacterial strains, media and culture conditions

Plasmids used in this study are listed in **Supplementary Table S1**. *Z. mobilis* TISTR 548 (Sootsuwan *et al*., 2007) was cultured in YPD medium [0.5% (w/v) yeast extract, 0.3% (w/v) peptone, and 3% (w/v) glucose]. Recombinant plasmids with targeted genes were introduced into *E. coli* DH5α (Toyobo, Japan) cells, and the cells were incubated in LB medium (1% tryptone, 0.5% yeast extract, and 0.5% NaCl) for 2 h and spread onto agar plates containing 1.5% agar. When pZA22 (Misawa et al., 1986) or its derivatives were introduced, agar plates were supplemented with chloramphenicol (50 $\mu g.ml^{-1}$). A log-phase culture, 0.5–1.0 at OD_{550}, that had been prepared at 30°C was inoculated into a liquid medium, and cultivation was conducted under a static condition. Bacterial growth was

monitored by measuring the optical density of the culture on a spectrophotometer (HITACHI, U-200) at OD_{550}. Determination of the CHT was carried out by the two-step cultivation assay as described previously (Kosaka *et al*., 2019). For measuring the cell length, 12-h cultivated cells in the first culture of the two-step cultivation were collected by low-speed centrifugation, washed, and resuspended in a saline solution. The morphology of the resuspended cells was then observed under a microscope (Eclipse E600, Nikon, Japan) with 400 × magnification, and images of the same sample were taken three to five times. The lengths of approximately 100 cells were manually measured.

2.3.3 Construction of expression plasmids of genes for RSEs and HSPs

Conventional recombinant DNA techniques were applied (Sambrook *et al*., 1989). For increased expression of genes for RSEs and HSPs in *Z. mobilis* TISTR548, operon fusion genes of the *pdc* promoter and each of these genes were constructed and incorporated into pZA22 (Misawa *et al*., 1986) as an expression vector for *Z. mobilis*. The *pdc* promoter fragment including the Shine–Dalgarno sequence of *pdc* (514 bp) and each gene fragment from its initiation codon to 20 bp downstream from its stop codon were amplified by PCR using the genomic DNA of TISTR548 as a template. pZA22 was linearized by PCR. The primers (**Supplementary Table S2**) used for PCR were designed according to the In-Fusion HD cloning method. The PCR fragments were purified using a QIAquick gel extraction kit and connected by an In-Fusion HD cloning kit. The constructed plasmids were confirmed by PCR and restriction mapping.

2.3.4 RT-PCR

To examine the degree of increased expression of targeted genes, RT-PCR was performed as described previously (Murata *et al*., 2011, 2018). Precultured cells were inoculated and cultivated in YPD medium containing chloramphenicol at 30°C for 12 h. The cells were then harvested, and total RNA was prepared by the hot phenol method (Aiba *et al*., 1981). RT-PCR was performed using a One-Step RNA PCR Kit with two specific primers for each gene (**Supplementary Table S3**) and 0.5 μg of total RNA according to the protocol from the kit supplier. After RT reaction at 50°C for 30 min, PCR consisting of denaturing at 94°C for 30 s, annealing at 50°C for 30 s, and extension at 72°C for 1 min was carried out. The PCR products after 10, 15, 20, and 25 cycles for each gene were taken and analyzed by 1.2% agarose gel electrophoresis, followed by staining with ethidium bromide. The intensity of bands of RT-PCR products was quantitatively determined using ImageJ. The linearity of the amplification was observed up to the 25th or 35th cycle. Under our conditions, the RT-PCR was able to specifically detect mRNA because no band was observed when reverse transcriptase was omitted.

2.3.5 Determination of ROS

The level of intracellular ROS was determined using a fluorescence probe, 2 ,7 - dichlorofluorescin diacetate (H_2DCFDA) (Pérez-Gallardo *et al*., 2013), as described previously (Kosaka *et al*., 2019). Cells that were grown for 12 h at 38°C in the first culture of the two-step cultivation assay were mixed with H2DCFDA at the final concentration of 5 μM, incubated for 30 min, and collected as a pellet by centrifugation (14,000 rpm) for 1 min. The pellet was then washed with saline solution, resuspended in 10 mM potassium phosphate buffer (pH 7.0), and disrupted by sonic oscillation (Cosmo Bio Japan). The fluorescence was

measured using a POWERSCAN® HT microplate reader (DS Pharma Biomedical Osaka Japan) with excitation at 485 nm and emission at 582 nm. Emission values were normalized by protein concentration, which was determined by the Lowry method (Dulley and Grieve, 1975).

2.3.6 Observation of stress resistance

To examine the effects of increased expression of genes on resistance to stresses other than high temperatures, cells were precultured at 30° C until a mid-log phase, diluted (10^0 –10^4), and spotted on YPD agar plates supplemented with 6–2% glucose, 3–5% ethanol, or 0.03–0.3% acetic acid. The plates were then incubated at 30°C for 48 h. To examine the effect of H_2O_2 on cell growth, precultured cells were inoculated and cultivated at 30°C in YPD liquid medium containing H_2O_2 at a final concentration of 0.1 mM. The optical density of the culture at OD_{550} was then measured at 12 h. All experiments were triplicated.

2.4 Results

2.4.1 Effects of increased expression of genes for RSEs and HSPs on growth at the CHT

To examine the contribution of RSEs and HSPs to the survival of *Z. mobilis* at the CHT and to the improvement of the CHT, the genes coding for these enzymes and proteins (**Table 2.1**) were individually cloned into pZA22 under the control of the *pdc* promoter, which is a relatively strong promoter derived from *Z. mobilis*, and the effects of expression of these genes were evaluated by the two-step cultivation assay, which enables determination of the CHT of mesophiles (Kosaka *et al*., 2019). The expression of cloned genes was confirmed by RT-PCR, indicating 1.4-to 4.0-fold increases compared to the expression of intrinsic genes

in the genome (**Supplementary Figure S1**). Two-step cultivation assays for RSE genes were conducted at 37.5, 38, 38.5, and 39°C (**Figure 2.1**). At 38°C, all transformants with pZA-Ppdc-sod, pZA-Ppdc-cat, pZA-Ppdc-cyt, pZA-Ppdc-ahpC1, pZA-Ppdc-ahpC2, or pZA-Ppdc-ZMO1573, which bear *sod*, *cat*, *cyt*, *ahpC1*, *ahpC2* [corresponding to *ahpC* (Charoensuk *et al*., 2011)], or *ZMO1573*, respectively, exhibited growth in the second culture, but the transformant with an empty vector as a control hardly grew. Among the genes tested, *sod*, *cat*, *ZMO1573*, and *ZZ6-0186* had relatively stronger effects on cell growth, especially after 36 h. At 38.5°C, transformants with pZA-Ppdc-sod, pZA-Ppdc-cat, pZA-Ppdc-ZMO1573, or pZA-Ppdc-ZZ6-0186 showed clearly stronger growth than those with pZA-Ppdc-cyt, pZA-Ppdc-ahpC1, pZA-Ppdc-ahpC2, or pZA-Ppdc-ZMO1573 in the second culture. The two-step cultivation data indicated that the CHTs of transformants with pZA-Ppdc-sod, pZA-Ppdc-cat, pZA-Ppdc-ZMO1573, or pZA-Ppdc-ZZ6-0186 were 38.5°C and that the CHTs of the remaining transformants were between 38 and 38.5°C. The improvement of CHT in the former group was 1°C compared to the CHT, 37.5°C, of the transformant with an empty vector and that of the latter was more than 0.5°C and less than 1°C. These findings suggest that all of the tested genes are able to improve the CHT of *Z. mobilis* TISTR 548 and that *sod*, *cat*, and *ZMO1573* are more effective.

The effects of increased expression of genes for HSPs were also examined by two-step cultivation in the range of 37.5–39°C, in which transformants with pZA-Ppdc-degP, pZA-Ppdc-dnaKJ, pZA-Ppdc-groELS, pZA-Ppdc-hsp20, pZA-Ppdc-hisU, pZA-Ppdc-clpB, or pZA-Ppdc-clpPX, which bear *degP*, *dnaKJ*, *groELS*, *hsp20*, *hisU*, *clpB*, or *clpPX*, respectively, were used (**Figure 2.2**). At 38°C, all transformants except for a transformant with pZA-Ppdc-degP exhibited higher growth in the second culture than the growth of the transformant with an empty vector. Of these, transformants with pZA-Ppdc-dnaKJ, pZA-

Ppdc-Hsp20, pZA-Ppdc-clpB, pZA-Ppdc-clpA, or pZA-Ppdc-clpS exhibited higher turbidity than that of the other transformants. On the other hand, cells containing pZA-Ppdc-degP showed only slightly higher turbidity than that of cells harboring an empty vector. At 38.5°C, in comparison with cells harboring an empty vector, cells harboring pZA-Ppdc-dnaKJ, pZA-Ppdc-Hsp20, or pZA-Ppdc-clpS showed relatively high turbidity in the second culture, followed by cells harboring pZA-Ppdc-groELS, pZA-Ppdc-clpB, or pZA-Ppdc-clpA. In the case of transformants with pZA-Ppdc-hslU or pZA-Ppdc-hsp33, their growth in the second culture was greatly repressed until 36 h compared to the control, suggesting that overexpression of *hslU* or *hsp33* somehow hampers the cell growth. Notably, the transformant with pZA-Ppdc-hsp33 showed lower turbidity even in the first culture. These findings suggested that increased expression of all of the genes tested except for *degP*, *hslU*, and *hsp33* can improve the thermotolerance of *Z. mobilis* TISTR 548 and that *dnaKJ*, *hsp20*, and *clpS* can up-shift the CHT by 1°C and *groELS*, *clpB*, and *clpA* can up-shift the CHT by 0.5–1°C.

Table 2.1 Genes for reactive oxygen species (ROS)-scavenging enzymes (RSE) and heat shock proteins (HSPs) that were examined in this study

Genes		Quary coverage[a] (%)	Identitiy[a] (%)	Function	Reference
Z. mobilis	***E. coli***				
RSE's genes					
sod	*sodB*	98	52	Superoxide dismutase, Fe-Mn family	Davidson et al., 1996
cat	*katE*	51	49	Catalase	Davidson et al., 1996
cyt	*yhjA*	96	44	Predicted cytochrome C peroxidase	Charoensuk et al., 2011
ZZ6_1529	*yfeX*	89	36	Dyp-type peroxidase family	Matsushita et al., 2015
ahpC1	Not applicable			ahpC/TSA family protein	La Carbona et al., 2009
ahpC2	*ahpC*	88	38	Alkyl hydroperoxide reductase subunit C	Seaver and Imlay, 2001
ZZ6_0186	*trxB*	95	55	Thioredoxin reductase	William, 1995; Arner and Holmgen, 2000
HSP's genes					
degP	*degP*	92	34	Serine protese Do	Jones et al., 2002; Murata et al., 2011; Charoensuk et al., 2017
dnaK	*dnaK*	99	66	Molecular chaperone DnaK	Tomoyasu et al., 1998
dnaJ	*dnaJ*	98	52	Molecular chaperone DnaJ	Tomoyasu et al., 1998
groEL	*groEL*	96	67	Chaperonin GroEL	Tomoyasu et al., 1998
groES	*groES*	97	52	Chaperonin GroES	Tomoyasu et al., 1998
hslU	*hslU*	98	53	ATP-dependent HslUV protease, ATP binding subunit HslU	Seol et al., 1997; Yoo et al., 1998;
ibpA	*ibpA*	99	50	Heat shock protein Hsp20	Kitagawa et al., 2002; Kuczynska-Wisnik et al., 2002
clpA	*clpA*	97	59	ATP-dependent Clp protease, ATP-binding subunit ClpA	Katayama et al., 1988; Kress et al., 2009
clpB	*clpB*	99	58	ATP-dependent Clp protease, ATP-binding subunit ClpB	Mogk et al., 2003; Kedzierska, 2003
clpS	*clpS*	92	55	ATP-dependent Clp protease, adaptor protein ClpS	Dougan et al., 2000; Roman-Hernandez et al., 2011
ZZ6_0844	*hslO*	91	33	Molecular chaperone Hsp33	Jakob et al., 1999

[a]Query coverage and identity of each gene were compared with *Z. mobilis* TISTR548 and *E. coli* W3110

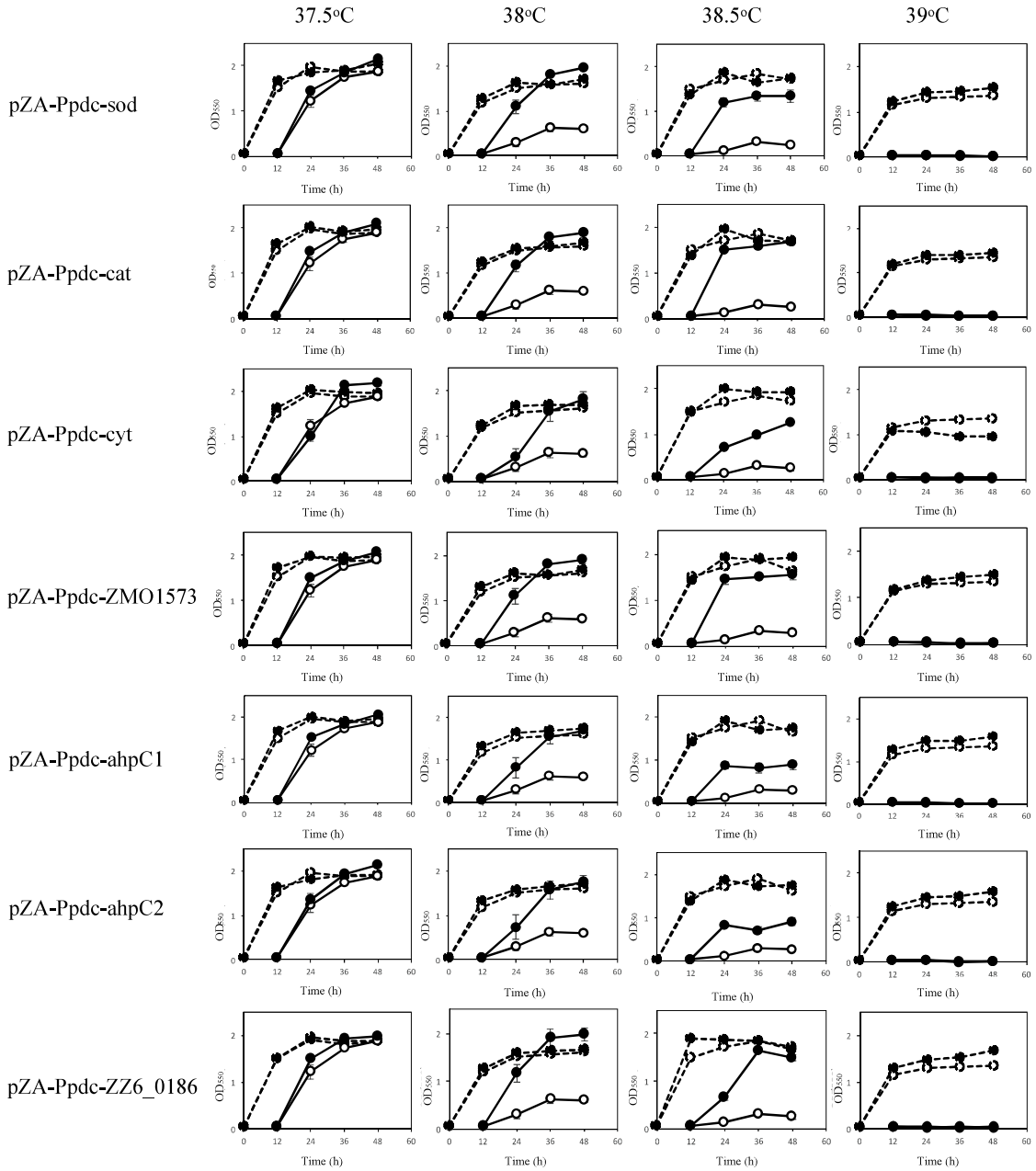


Figure 2.1. Effects of increased expression of genes for reactive oxygen species (ROS)-scavenging enzymes (RSEs) on growth at the critical high temperature (CHT). Growth of cells harboring a recombinant plasmid with a gene for one of the RSEs (closed circles) or an empty plasmid (open circles) was examined by two-step cultivation at 37.5–39°C in YPD medium containing chloramphenicol. After 12 h in the first cultivation (dotted lines) at each temperature, an aliquot of the culture was transferred to a fresh medium, and the second cultivation was carried out (straight lines). At the times indicated, cell density was estimated by measuring OD_{550}. Bars represent ± SD for three independent experiments.

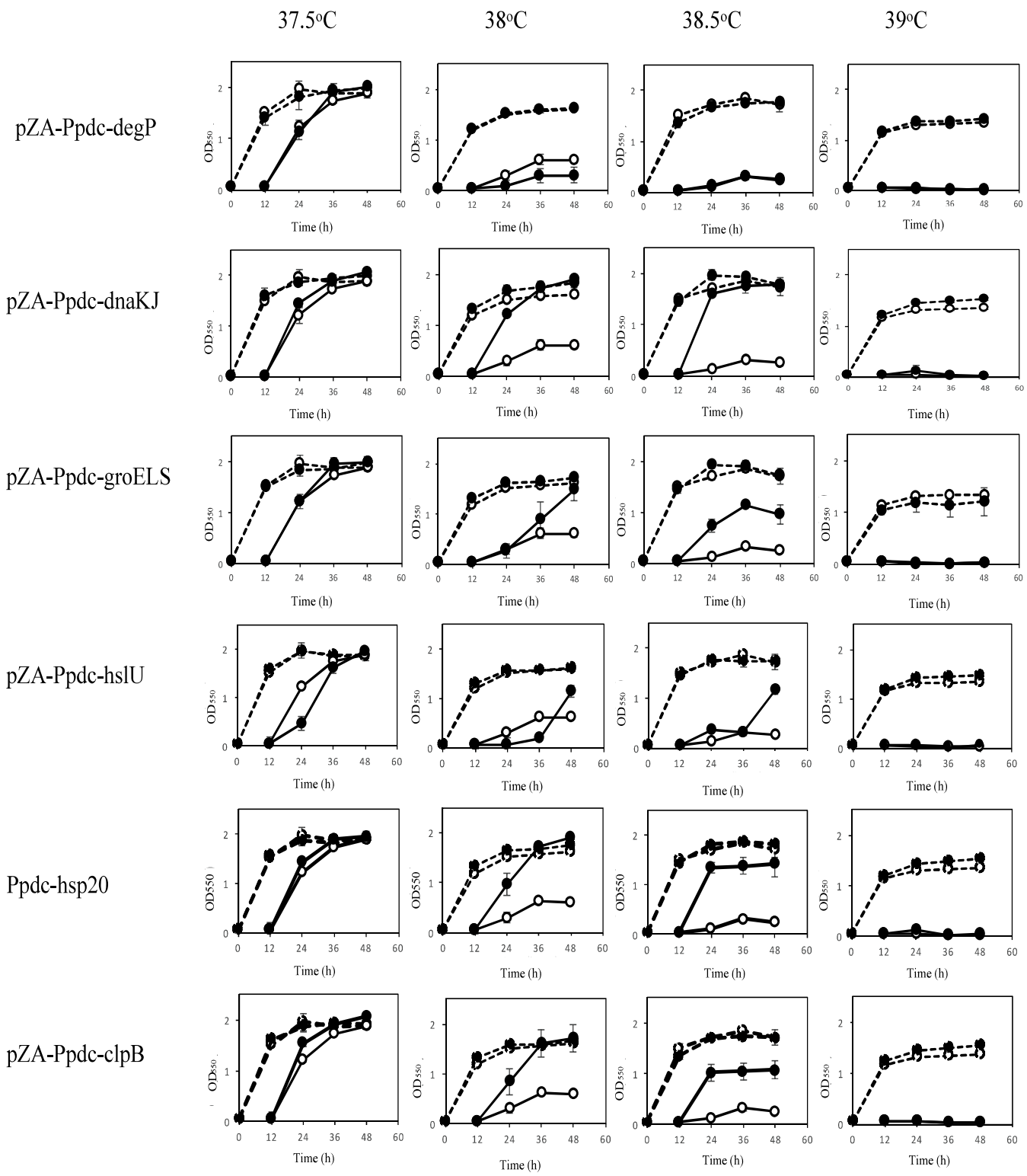

37.5°C
38°C
38.5°C
39°C
pZA-Ppdc-degP
pZA-Ppdc-dnaKJ
pZA-Ppdc-groELS
pZA-Ppdc-hslU
Ppdc-hsp20
pZA-Ppdc-clpB
OD550
Time (h)

Figure 2.2. Continued

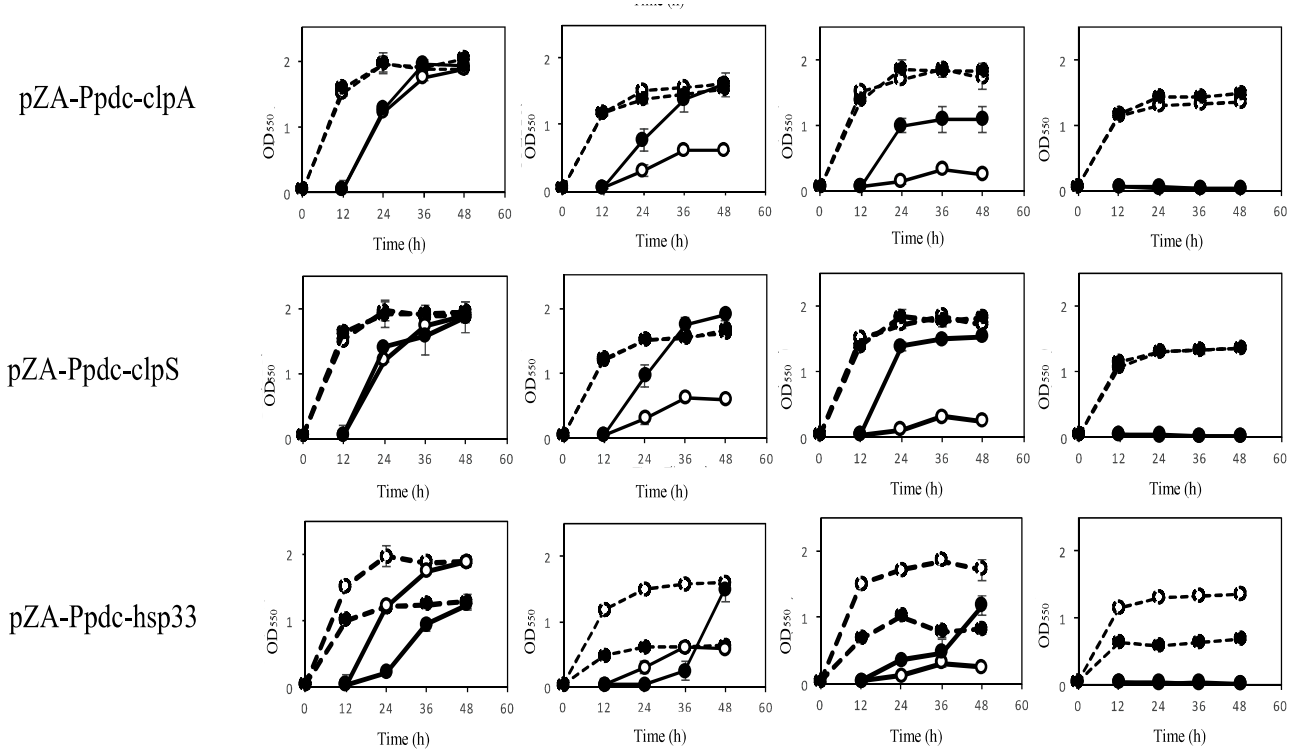


Figure 2.2. Effects of increased expression of genes for heat shock proteins (HSPs) on growth at the critical high temperature (CHT). Growth of cells harboring a recombinant plasmid with a gene for one of the HSPs (closed circles) or an empty plasmid (open circles) was examined by two-step cultivation at 37.5–39°C in YPD medium containing chloramphenicol as shown in **Figure 2.1**. Bars represent ± SD for three independent experiments.

2.4.2 Effects of increased expression of genes for RSEs and HSPs on accumulation of ROS at the CHT

When cells are exposed to a temperature close to the CHT, the level of intracellular ROS increases (Kosaka *et al*., 2019). Since enhancement of the expression of genes for RSEs and HSPs caused an up-shift of the CHT, it was assumed that increased expression of these genes prevented the accumulation of ROS. We thus examined the assumption at 38°C after 12 h in the first culture (**Figure 2.3)** because accumulation of ROS in the first culture has been reported to have an impact on the growth of cells in the second culture (Kosaka *et al*., 2019). As a result, the level of ROS of all of the transformants with a plasmid bearing one of the genes for RSEs and HSPs was lower than that of the control. However, the ROS level at the mid-log phase in the first culture was not always consistent with the level of improvement in the CHT because transformants with pZA-Ppdc-dnaKJ and pZA-Ppdc-clpA showed the lowest level of ROS but improved the CHT by 1°C and less than 1°C, respectively. This inconsistency may be due to other contribution of the genes, especially HSP genes, to the up-shift of the CHT in addition to the reduction of ROS.

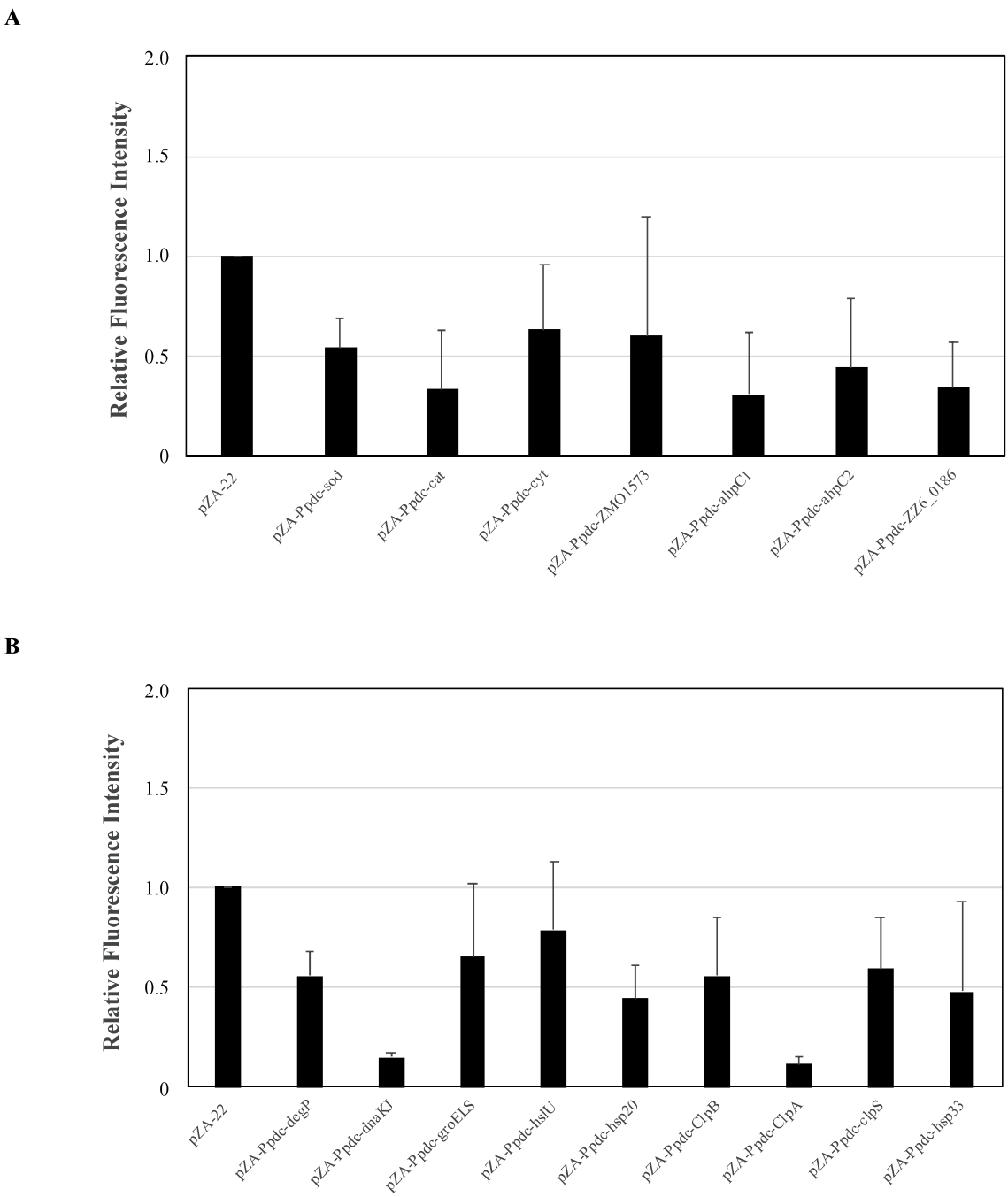


Figure 2.3. Effects of increased expression of genes for reactive oxygen species (ROS)-scavenging enzymes (RSEs) **(A)** and heat shock proteins (HSPs) **(B)** on accumulation of ROS at the critical high temperature (CHT). Cells were grown at 38°C in YPD medium containing chloramphenicol. Using the culture at 10 h, ROS were detected with H_2DCFDA, and the fluorescent intensity reflects the level of accumulation of ROS. Bars represent ± SD for three independent experiments.

2.4.3 Effects of increased expression of genes for RSEs and HSPs on cell morphology at the CHT

Cells become elongated when exposed to a temperature close to the CHT (Kosaka *et al*., 2019). The morphological change may be due to accumulation of stress including stress caused by ROS in cells, which presumably influences cell division. As mentioned above, the enhanced expression of genes for RSEs and HSPs reduced the intracellular level of ROS. We thus assumed that their enhanced expression prevents the morphological change. The assumption was examined by measurement of cell length at 38°C after 12 h in the first culture (**Figure 2.4**). As expected, all transformants with a plasmid bearing one of the genes for RSEs were shorter in cell length than the transformant with an empty vector, being consistent with the impact of the genes on cell growth at the CHT. While the transformants with *dnaKJ*, *groELS*, *clpB*, *clpA*, *clpS*, and *hsp33* for HSPs were relatively short in cell length, the transformant with *hsp20* showed no change, and the transformant with *degP* or *hslU* were much larger. Considering the lower levels of ROS in the transformants, increased expression of the latter three genes may somehow hamper cell division.

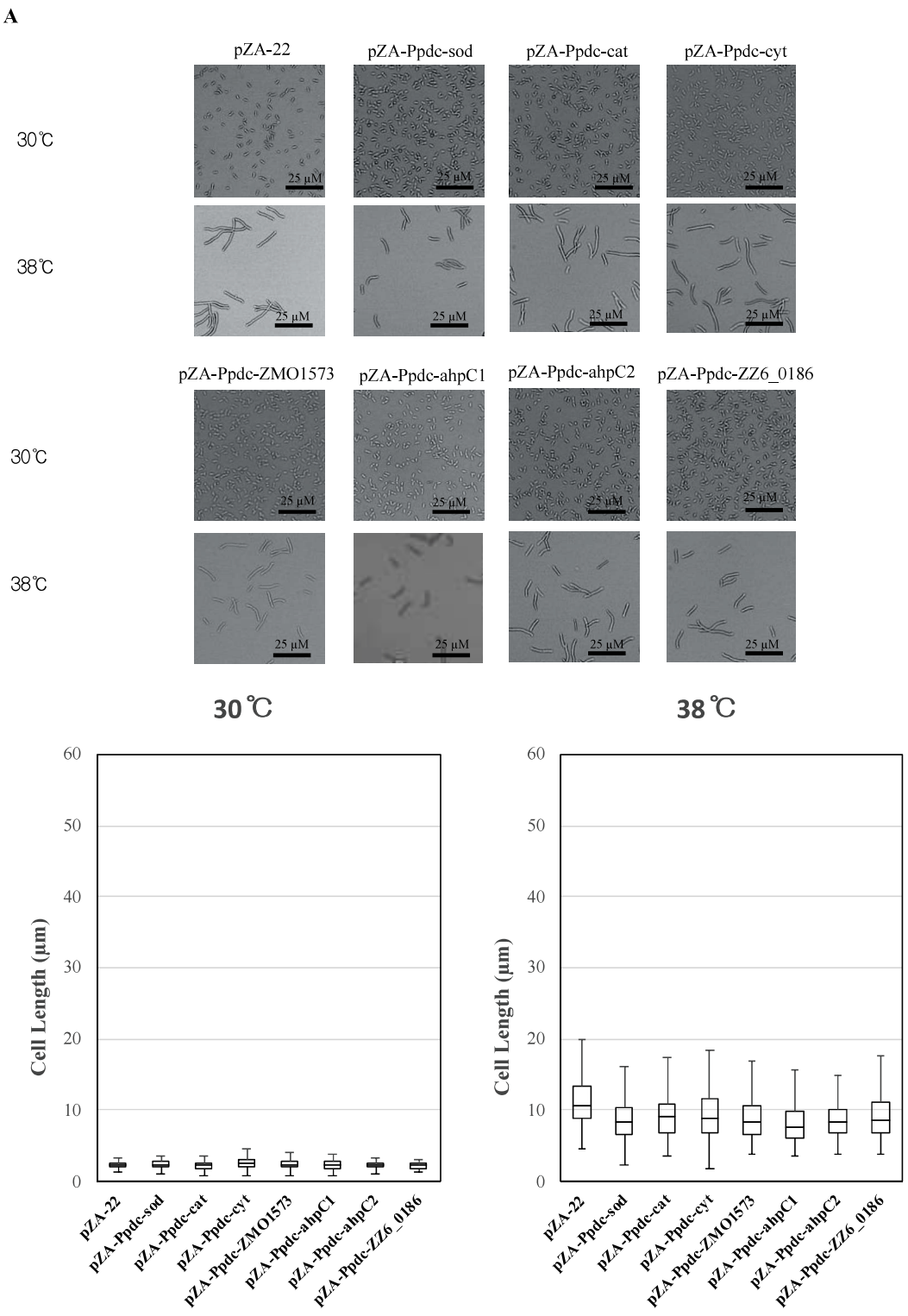


Figure 2.4. Continued

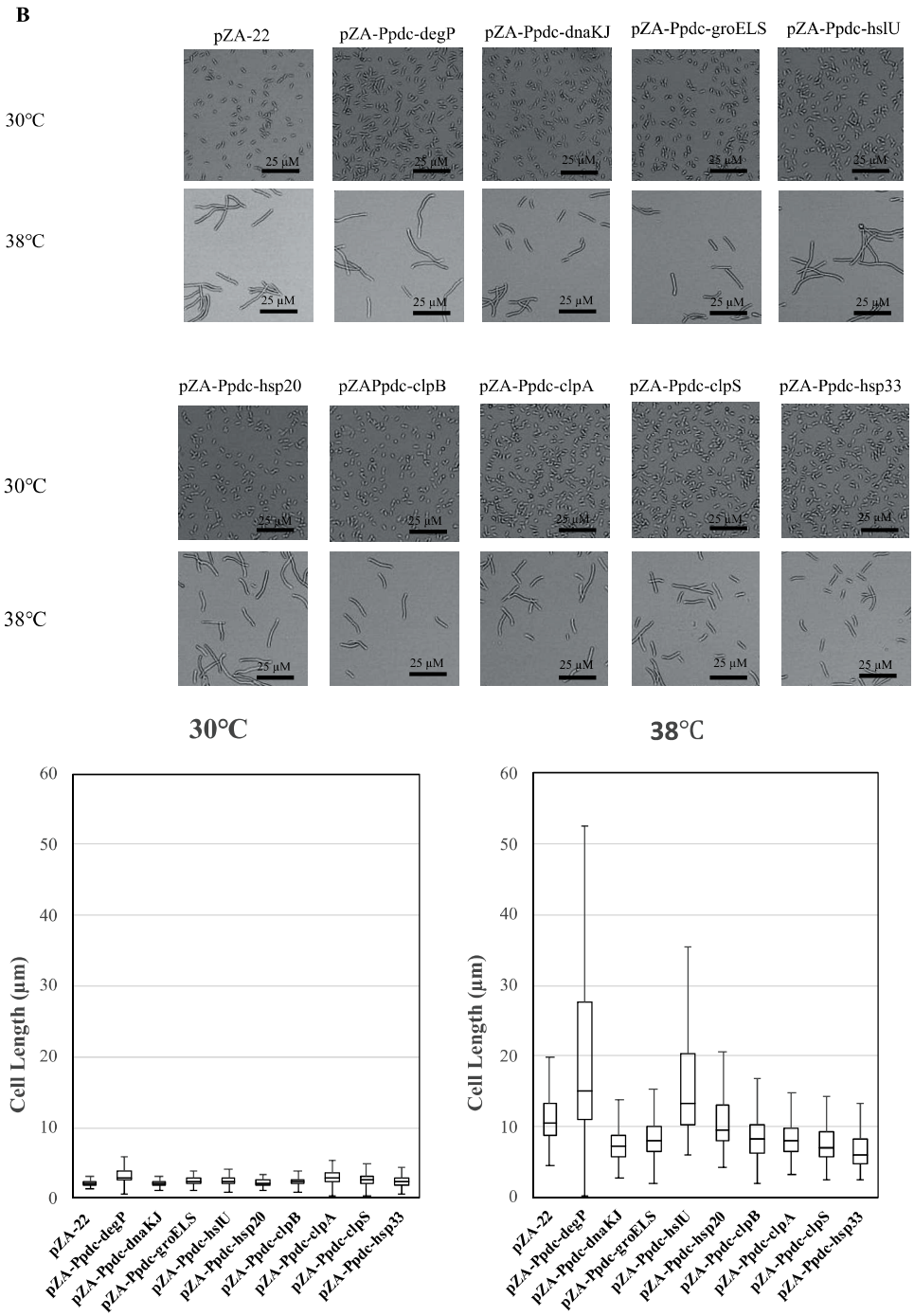


Figure 2.4 Effects of increased expression of genes for reactive oxygen species (ROS)-scavenging enzymes (RSEs) **(A)** and heat shock proteins (HSPs) **(B)** on cell morphology at the critical high temperature (CHT). Cells were grown at 38°C in YPD medium containing chloramphenicol. Using the culture at 12 h, cell morphology was observed, and lengths of 100 cells were measured.

2.4.4 Effects of increased expression of genes for RSEs and HSPs on growth under condition of stress

The experiments described above, in which the effects of increased expression of genes for RSEs and HSPs were examined, revealed that most of the genes were effective for improvement of the CHT, accumulation of ROS, and change in cell morphology. We thus examined whether these genes allow cells to be resistant to various types of stress in addition to heat (**Figure 2.5** and **Supplementary Figure S2**). When tested on plates containing different concentrations of glucose or ethanol, no transformant with any gene for RSEs and HSPs showed growth that was different from that of the control transformant. After addition of 0.03% acetic acid (about pH 5.0) and 0.3% acetic acid (about pH 4.0), all transformants including those with an empty vector grew better than those without the addition of acetic acid, which may be because the host strain has the optimal pH in the acidic range. Interestingly, transformants with some genes of RSEs, *sod*, *cat*, *ahpC1*, *ahpC2*, and *ZZ6_0186*, and transformants with some genes of HSPs, *dnaK*, *hsp20*, *clpA*, *clpB*, and *clpS*, exhibited better growth on a medium containing acetic acid, being almost consistent with their up-shift of the CHT. Therefore, it is likely that the presence of acetic acid causes the macromolecule damage by a similar mechanism to that at the CHT, but it might be more than such damage when cells are challenged by acetic acid, for example, ATP deprivation and lower pH inside the cell (Lawford and Rousseau, 1993; Ullah *et al*., 2013). Moreover, exogenous oxidative stress was examined by cultivation at 30°C in YPD liquid medium containing 0.1 mM H_2O_2. Most of the transformants with genes for RSEs and HSPs except for *degP* showed better growth than that with an empty vector (**Supplementary Figure S3**), suggesting that increased expression of these genes alleviated the oxidative stress by H_2O_2.

These findings are essentially consistent with the results of measurement of ROS in these transformants, as shown in **Figure 2.3**.

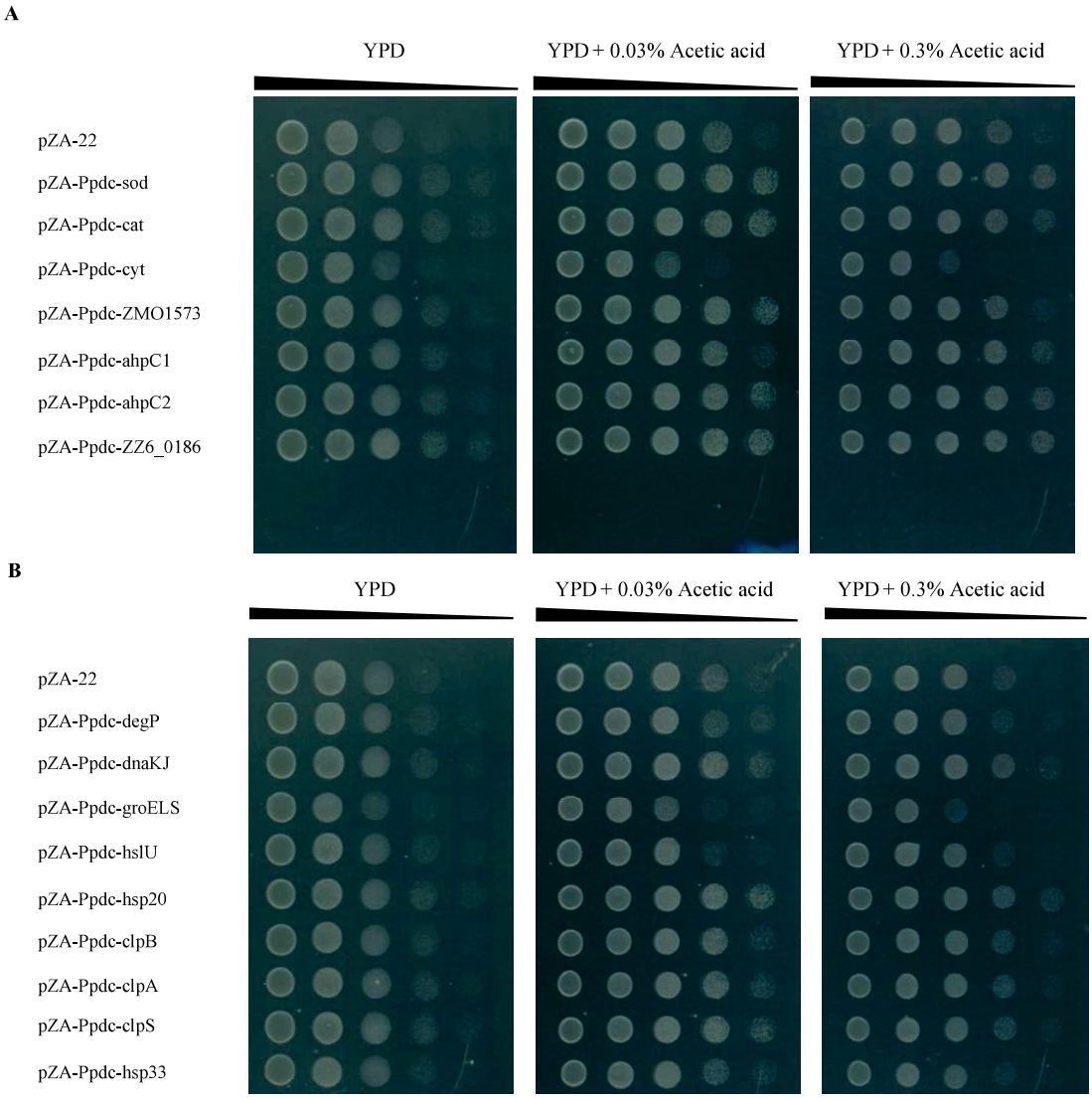


Figure 2.5. Effects of increased expression of genes for reactive oxygen species (ROS)-scavenging enzymes (RSEs) **(A)** and HSPs **(B)** on tolerance to acetic acid. Cells were grown at 30°C in YPD medium containing chloramphenicol overnight. The cell culture was serially diluted, spotted on YPD agar plates containing 0.03 or 0.3% acetic acid, and incubated at 30°C for 48 h.

2.5 Discussion

When exposed to a CHT, mesophiles exhibit several characteristic phenotypes, including cell elongation and accumulation of ROS (Kosaka *et al*., 2019). To determine the CHT of mesophiles, we had developed a two-step cultivation assay (Kosaka *et al*., 2019), which is much clearer and more precise than a general cultivation assay that shows an increase in turbidity even at a temperature above the CHT. On the other hand, a temperature around the CHT may cause instability of the membrane, resulting in leakage of electrons to generate ROS and may give rise to unfolding or denaturing of proteins or oxidation of proteins by accumulated ROS (Jan-Ulrik *et al*., 2015). In this study, we thus applied the two-step cultivation method to evaluate the contribution of genes for RSEs and HSPs to the CHT of *Z. mobilis* TISTR548. Their contribution was further examined by observation of the levels of ROS accumulation and cell elongation.

Among the genes tested, enhanced expression of *sod*, *cat*, *ZMO1573*, and *ZZ6-0186* for RSEs and *dnaKJ*, *hsp20*, and *clpS* for HSPs up-shifted the CHT by 1°C, and most of the remaining genes up-shifted the CHT by 0.5–1.0°C. However, the contribution of these genes is less than the effect of thermal adaptation, which is able to increase the CHT by 2–3°C (Kosaka *et al*., 2019). Consistent with the weaker contribution to the up-shift of the CHT, the extents of reduction of ROS and of cell size seem to be relatively low. Nonetheless, it is likely that prevention of the accumulation of ROS is an effective way to improve the CHT.

The HSP genes tested in this study except for *degP*, *hslU*, and *hsp33* were able to raise the CHT of *Z. mobilis* when their expression was increased (**Figure 2.2**). DnaKJ and GroELS function as molecular chaperones, which are involved in refolding of unfolded or denatured proteins (Narberhaus *et al*., 1998; Tomoyasu *et al*., 1998; Thanonkeo *et al*., 2007; Al Refaii and Alix, 2009). Hsp20 and ClpB as Hsp100 members prevent protein aggregation

and solubilize aggregated proteins, respectively (Zolkiewski *et al*., 2012; Mogk *et al*., 2015). These chaperones or Hsps deal with denaturated or aggregated proteins that are expected to appear at the CHT. ClpA and ClpS are ATP- binding subunits of ClpAP protease and its adapter, respectively (Dougan *et al*., 2002; Maglica *et al*., 2008). They may contribute to the removal of denatured or aggregated proteins at the CHT. On the other hand, increased expression of *hslU* and *hsp33* seems to inhibit cell growth even at 37.5 and 38°C. The former product is an ATP-binding subunit of HslUV protease (Yoo *et al*., 1998; Baytshtok *et al*., 2017), and the latter is a redox-regulated chaperone, which is activated by dimerization *via* disulfide bonds (Graf and Jakob, 2002). Considering these functions and activation process, the negative effects of HslU and Hsp33 on growth at the CHT might be due to proteolysis of and abnormal interaction with crucial proteins for cellular activities. In contrast to the HSP genes tested except for *hslU* and *hsp33*, *degP* for a periplasmic chaperone hardly elevated the CHT when overexpressed. A knockout mutant of *degP*, however, reduced the CHT by 1–2°C, and the gene has thus been categorized as a thermotolerant gene, which is shared by *E. coli*, *Z. mobilis*, and *A. tropicalis* (Murata *et al*., 2011, 2018). The inconsistency between *degP* and effective HSP genes may be due to the different cellular localization of their gene products in cells: DegP localizes in the periplasmic space, but other gene products localize in the cytoplasm. Alternatively, DegP might be sufficient to perform its activity in the parental strain. The increased expression of most of the cytoplasmic HSPs, but not DegP, may thus contribute to the maintenance of homeostasis inside cells at a CHT.

All seven of the RSE genes tested decreased the level of ROS and increased the CHT of *Z. mobilis* by their increased expression (**Figures 2.1**, **2.3**). Four of the genes are involved in degradation of H_2O_2, but regulation of their expression may be distinct under different temperature conditions: the expression levels of *cytC* and *ZMO1573* are higher at

37°C than at 30°C, whereas *cat* and *ahpC* exhibit the opposite expression pattern to that of *cytC* and *ZMO1573* (Charoensuk *et al*., 2011). Such up-regulation of *cytC* and *ZMO1573* at a high temperature may reflect insufficient H_2O_2 -degrading activities for survival at the CHT, being in agreement with the finding that all of the four genes are able to raise the CHT when overexpressed. On the other hand, the reduced form of thioredoxin plays an important role as an antioxidant, and its reduction requires NADPH in addition to the corresponding reductase. It is likely that NADPH is not limited in *Z. mobilis* cells at the CHT because increased expression of *ahp1* for peroxiredoxin or *ZZ6_0186* for thioredoxin reductase leads to an increase in the CHT.

This study was motivated by the surprising fact that only a few genes for RSEs and HSPs have been identified as thermotolerant genes (Charoensuk *et al*., 2017; Murata *et al*., 2018), which are essential for survival at a CHT. The finding presented suggests that many genes for RSEs and HSPs have the potential to improve the CHT, although the range of improvement is within 1°C. It is thus likely that there are several genes that have overlapping functions in cells, and thus, the disruption of one gene is complemented by another gene. In relation to this, a knockout mutant of *cytC* for a peroxidase involved in the respiratory chain exhibited filamentous shapes and reduction in growth under a shaking condition at a high temperature, and under the same condition, *sod*, *ahpC*, and *ZMO1573* are complementarily expressed to the *cytC* mutation (Charoensuk *et al*., 2011). Such robustness by the existence of complementing genes would have hindered the identification of genes for RSEs and HSPs as thermotolerant genes.

CHAPTER 3

Enhancement of Thermal Resistance by Metal Ions in Thermotolerant *Zymomonas mobilis* TISTR 548

3.1 Abstract

The thermal resistance of fermenting microbes is a key characteristic of stable fermentation at high temperatures. Therefore, the effects of various metal ions on the growth of *Zymomonas mobilis* TISTR 548, a thermotolerant ethanologenic bacterium, at a critical high temperature (CHT) were examined. Addition of Mg^{2+} and K^{+} increased CHT by 1°C, but the effects of the addition of Mn^{2+}, Ni^{2+}, Co^{2+}, Al^{3+}, Fe^{3+}, and Zn^{2+} on CHT were negligible. To understand the physiological functions associated with the addition of Mg^{2+} or K^{+}, cell morphology, intracellular reactive oxygen species (ROS) level, and ethanol productivity were investigated at 39°C (i.e., above CHT). Cell elongation was repressed by Mg^{2+}, but not by K^{+}. Addition of both metals reduced intracellular ROS level, with only K^{+} showing the highest reduction strength, followed by both metals and only Mg^{2+}. Additionally, ethanol productivity was recovered with the addition of both metals. Moreover, the addition of Mg^{2+} or K^{+} at a non-permissive temperature in 26 thermosensitive, single gene-disrupted mutants of *Z. mobilis* TISTR 548 revealed that several mutants showed metal ion-specific growth improvement. Remarkably, K^{+} repressed growth of two mutants. These results suggest that K^{+} and Mg^{2+} enhance cell growth at CHT via different mechanisms, which involve the maintenance of low intracellular ROS levels.

3.2 Introduction

Recently, bioethanol has gained attention as an alternative to fossil fuel because as a carbon-neutral fuel, it can potentially delay the progress of global warming (Hahn-Hägerdal *et al*., 2006; Chisti, 2008). However, industrial scale bioethanol production requires a more cost-effective process to be economically competitive. High-temperature fermentation (HTF; which enables fermentation at a temperature 5–10°C higher than that used in the conventional process) may reduce (1) cooling cost, (2) running cost at the simultaneous saccharification and fermentation stage, and (3) contamination risks (Abdel-Banat *et al*., 2010; Kosaka *et al*., 2018). Bioethanol production by HTF requires high-efficiency ethanol production and thermotolerant microorganisms. *Zymomonas mobilis*, a Gram-negative, facultative, anaerobic bacterium, performs high-speed ethanol production (He *et al*., 2014) compared with the conventional ethanol producer *Saccharomyces cerevisiae*, whose cultivation temperature of >35°C is not permissible for yeast growth (Aldiguier *et al*., 2004). *Z. mobilis*, which uses the Entner–Doudoroff pathway and an incomplete TCA cycle, is facultatively anaerobic and requires no oxygen for its growth; conversely, it assimilates glucose, fructose, and sucrose as the sole carbon sources (Panesar *et al*., 2006; He *et al*., 2014). We focused on *Z. mobilis* TISTR 548, one of the thermotolerant *Z. mobilis* strains that grew at 39°C (Sootsuwan *et al*., 2007) and developed thermotolerant mutants by thermal adaptation enhancement of its critical high temperature (CHT), an upper limit for survival, up to 2°C (Matsushita *et al*., 2016; Kosaka *et al*., 2019). We subsequently used this mutant strain with HTF using a model fermentation and distillation system to reveal the effectiveness of this method and bioethanol productivity by HTF with *Z. mobilis* (Murata *et al*., 2015).

Metal ions enhance the growth of ethanologenic microorganisms at CHT. Microorganisms require several ions as essential metals for the normal function and

homeostasis of a wide range of cellular proteins (Reid *et al*., 2009), but these ions are toxic at high concentrations (Gadd, 1992). Among these ions, only Mg^{2+} has been reported to enhance thermotolerance in *Z. mobilis* strains (Thanonkeo *et al*., 2007). Moreover, Mg^{2+} helped recover thermosensitive mutants of *Z. mobilis* TISTR 548, in which genes for membrane stabilization or membrane formation were disrupted, suggesting that at CHT, Mg^{2+} stabilizes membrane structure and protects cells from heat (Charoensuk *et al*., 2017). Mg^{2+} also stabilizes the outer membrane (OM) structure, at least of lipopolysaccharide (LPS), of cells by divalent cation crossbridging (bridging action) in Gram-positive bacteria (Nikaido, 2003). Studies on several microorganisms, particularly *Escherichia coli* (Murata *et al*., 2011) and *Lactobacillus* strains (Yang *et al*., 2017), have revealed the thermotolerance-enhancing effect of Mg^{2+}. However, although the enhancing effects of K^{+} and Ca^{2+} on *S. cerevisiae* (Lam *et al*., 2014) and lactic acid bacteria (Huang and Chen, 2013), respectively, have been reported, there is no report about the enhancement effects of these and other metals on *Z. mobilis* growth at CHT. This evidence suggests that the concentration of several metals in a fermentation medium is important for efficient HTF for bioethanol production. However, the effect of a wide range of metals on *Z. mobilis* TISTR 548 at CHT is yet to be investigated. Besides, the mechanism underlying the effects of these metals, such as Mg^{2+}, on cell physiology at CHT remains unclear.

In this study, the effects of metal ions, i.e., Mn^{2+}, Ni^{2+}, Co^{2+}, Al^{3+}, Fe^{3+}, Zn^{2+}, Mg^{2+}, K^{+}, and Ca^{2+}, on *Z. mobilis* TISTR 548 growth at CHT were observed. Moreover, the effects of Mg^{2+} and K^{+} (these metals enhanced growth at CHT) on the physiology of *Z. mobilis* TISTR 548 and its thermosensitive mutants were investigated.

3.3 Materials and methods

3.3.1 Bacterial strains, media, and cultivation conditions

The bacterial strains used in this study are listed in **Table 3.1**. To grow *Z. mobilis*, a preculture was prepared in 2 mL of YPD medium (0.3% yeast extract, 0.5% peptone, and 3% glucose) and incubated overnight at 30°C. The overnight culture was subsequently inoculated into fresh YPD medium at an OD_{550} of 0.05. Cultivation was performed under non-shaking (static) conditions.

3.3.2 Examination of the effects of various materials on cell growth

To compare the effects of additional reagents, cells were subjected to two-step cultivation (Kosaka *et al*., 2019) at the same temperature to observe the effect of temperature or additional reagents. Two-step cultivation can simply determine the temperature-upper limit for the survival of cells because when the first culture is performed at a temperature just above a CHT, cells cannot grow in the second culture at the same temperature (Kosaka *et al*., 2019). In the first culture, the OD value of the culture increases even at a temperature over CHT because of cell elongation. The CHT of *Z. mobilis* TISTR 548 has been determined to be 38°C by this method (Kosaka *et al*., 2019). Reagents were added to a medium at the desired condition before each inoculation. Briefly, the first cultivation was performed until the culture attained a late log phase at a temperature around a putative CHT; then, a portion of the first culture was transferred into a fresh medium at an OD_{550} of 0.05 and cultured at the same temperature. All metals tested were obtained in the form of chloride salts.

3.3.3 Cell morphology

Cell morphology was observed using phase-contrast microscopy (E6F-RFK-1, Nikon, Tokyo, Japan). In total, 100 cells were randomly selected on microphotographs, and their length was measured using ImageJ (Schneider *et al*., 2012).

Table 3.1 List of *Z. mobilis* strains used in this study

Strains	Genotypes	References or Sources
TISTR548		TISTR collections
TC01	TISTR548 (*ZZ6_0707*::Tn*10*)	Charoensuk *et al.*, 2017
TC03	TISTR548 (*ZZ6_1376*::Tn*10*)	Charoensuk *et al.*, 2017
TE12	TISTR548 (*ZZ6_1146*::Tn*10*)	Charoensuk *et al.*, 2017
C12-36	TISTR548 (*ZZ6_1551*::Tn*10*)	Charoensuk *et al.*, 2017
C11-44	TISTR548 (*ZZ6_1046*::Tn*10*)	Charoensuk *et al.*, 2017
C13-36	TISTR548 (*ZZ6_1210*::Tn*10*)	Charoensuk *et al.*, 2017
TC04	TISTR548 (*ZZ6_0923*::Tn*10*)	Charoensuk *et al.*, 2017
1-2	TISTR548 (*ZZ6_1043*::Tn*10*)	Charoensuk *et al.*, 2017
3-24	TISTR548 (*ZZ6_0929*::Tn*10*)	Charoensuk *et al.*, 2017
TC14	TISTR548 (*ZZ6_0158*::Tn*10*)	Charoensuk *et al.*, 2017
C31-23	TISTR548 (*ZZ6_1254*::Tn*10*)	Charoensuk *et al.*, 2017
TC15	TISTR548 (*ZZ6_1477*::Tn*10*)	Charoensuk *et al.*, 2017
F32	TISTR548 (*ZZ6_0616*::Tn*10*)	Charoensuk *et al.*, 2017
C12-43	TISTR548 (*ZZ6_0934*::Tn*10*)	Charoensuk *et al.*, 2017
TC10	TISTR548 (*ZZ6_0681*::Tn*10*)	Charoensuk *et al.*, 2017
C12-44	TISTR548 (*ZZ6_0023*::Tn*10*)	Charoensuk *et al.*, 2017
C21-17	TISTR548 (*ZZ6_1659*::Tn*10*)	Charoensuk *et al.*, 2017
TC05	TISTR548 (*ZZ6_0980*::Tn*10*)	Charoensuk *et al.*, 2017
TC12	TISTR548 (*ZZ6_0702*::Tn*10*)	Charoensuk *et al.*, 2017
TE19	TISTR548 (*ZZ6_0979*::Tn*10*)	Charoensuk *et al.*, 2017
C31-15	TISTR548 (*ZZ6_0019*::Tn*10*)	Charoensuk *et al.*, 2017
TC11	TISTR548 (*ZZ6_0840*::Tn*10*)	Charoensuk *et al.*, 2017
C12-37	TISTR548 (*ZZ6_0962*::Tn*10*)	Charoensuk *et al.*, 2017
TC09	TISTR548 (*ZZ6_0541:*:Tn*10*)	Charoensuk *et al.*, 2017
TC13	TISTR548 (*ZZ6_0861*::Tn*10*)	Charoensuk *et al.*, 2017
1-10	TISTR548 (*ZZ6_1289*::Tn*10*)	Charoensuk *et al.*, 2017

3.3.4 Intracellular reactive oxygen species Level

Zymomonas mobilis TISTR 548 cells were grown on YPD medium at 39°C. At 12 h, 5 μM H_2DCFDA was added to the first culture, and further cultivation was performed at 39°C for 30 min. Then, cells were harvested by low-speed centrifugation and washed once with phosphate-buffered saline [130 mM NaCl, 10.8 mM Na_2 HPO_4, 4.2 mM NaH_2 PO_4 (pH 7.2)]. The washed cells were disrupted by sonication for 30 min using an ultrasonic cell disruptor (Bioruptor; Cosmo Bio, Tokyo, Japan) and subjected to low-speed centrifugation. Supernatant fluorescence was measured using a microplate reader (POWERSCAN® HT; BioTek Instruments, Inc., Winooski, VT, United States). Protein concentration was determined using the Lowry method (Dulley and Grieve, 1975). The result obtained for intracellular reactive oxygen species (ROS) levels is expressed as fluorescence intensity per protein concentration, and the ratio of the number of cells grown in the presence of a metal ion to that of cells grown in its absence was estimated and expressed as percentage.

3.3.5 Ethanol concentration

Ethanol concentration was analyzed using a gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and Gaskuropack 54-packed glass column (60/80 mesh; GL Science, Tokyo, Japan); nitrogen was used as a carrier gas (flow rate, 35 mL/min). Operating temperatures were as follows: injection temperature, 200°C; column temperature, 180°C; and detector temperature, 200°C.

3.4 Results

3.4.1 Effects of metal ions on *Z. mobilis* TISTR 548 growth at CHT

To explore the metal ions that enhance *Z. mobilis* TISTR 548 growth at putative CHT, the growth levels with and without the addition of Ni^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+}, Mn^{2+}, Co^{2+}, Mg^{2+}, and K^{+} were compared. The effect of the addition of metal ions was evaluated with two-step cultivation, wherein only viable and culturable cells grow, whereas dead or viable but non-culturable cells do not grow in fresh medium at the second cultivation (Kosaka *et al*., 2019). At 38°C and 39°C, the growth level under the conditions of 0.01 mM $NiCl_2$, $ZnCl_2$, $FeCl_3$, $AlCl_3$, $MnCl_2$, and $CoCl_2$ was the same as that without the addition of metal ions (**Figure 3.1**). On the contrary, the addition of >0.1 mM $NiCl_2$ and $CoCl_2$ led to a lower growth level than no addition of metal ions at the first stage of cultivation (**Figures 3.1 A,B,K,L**). Similarly, the growth level following the addition of $ZnCl_2$ and $MnCl_2$ was lower at 1 mM (**Figures 3.1 C,D,I,J**). The growth trend did not change distinctly between 38 and 39°C (**Figure 3.1**). Ten millimolar $CaCl_2$ or 10 mM NaCl suppressed growth in the second step of *Z. mobilis* TISTR 548 cultivation at 38°C (data not shown). On the other hand, when $MgCl_2$ and KCl were added to the medium, there was a 1° higher growth than there was without adding metals even at 39°C (**Figures 3.2 A–C**). At 39.5 °C, there was negligible growth in the presence of $MgCl_2$ and KCl (**Figure 3.2D**). The results suggested that the optimum concentrations of $MgCl_2$ and KCl for growth enhancement at 39°C were 5 and 30 mM, respectively.

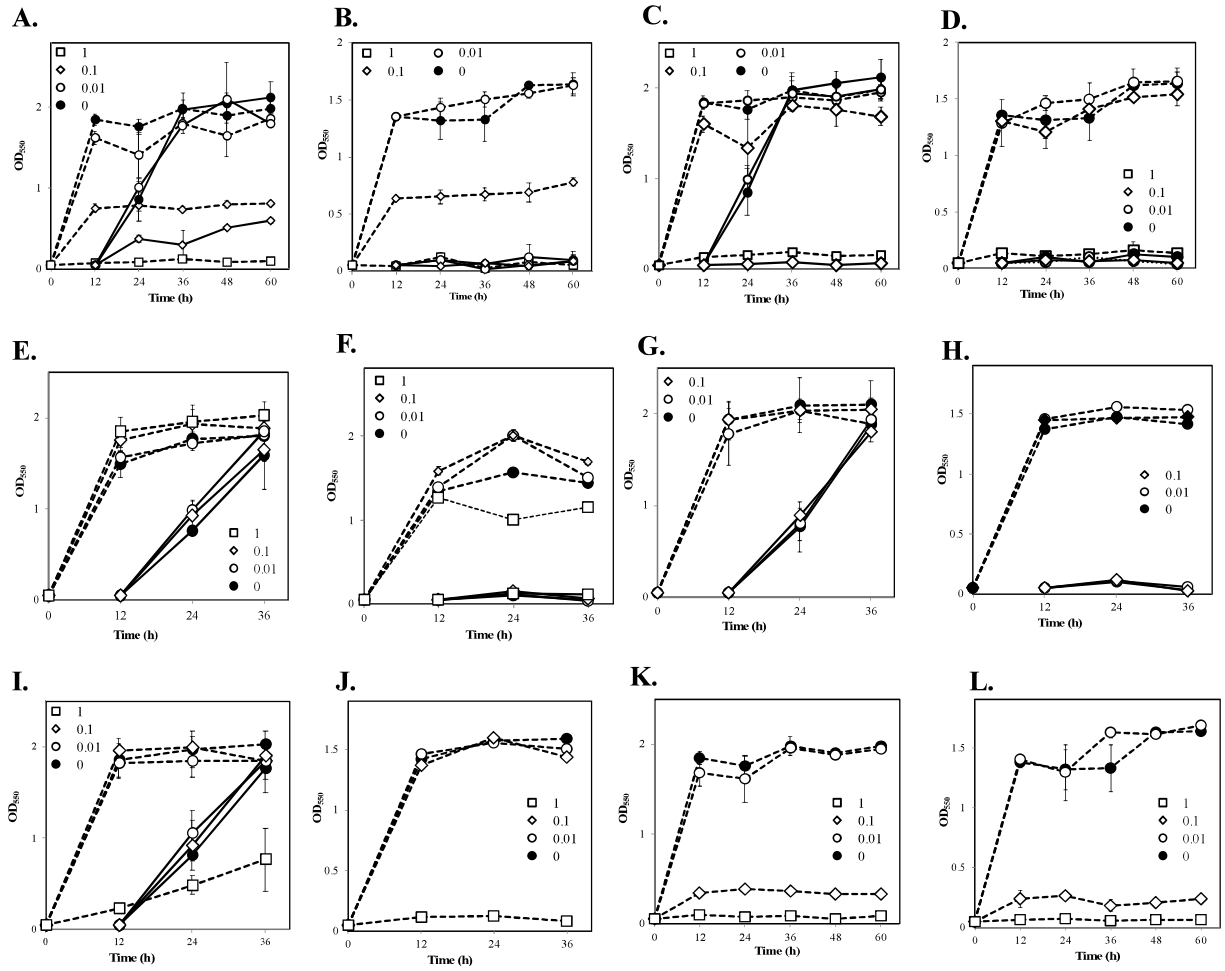


Figure 3.1 Effects of various metal ions on thr two-step cultivation of *Zymomonas mobilis* TISTR 548. Cells were cultivated in YPD medium at 38ₒC with different concentrations of $NiCl_2$ **(A)**, $ZnCl_2$ **(C)**, $FeCl_3$ **(E)**, $AlCl_3$ **(G)**, $MnCl_2$ **(I)**, or $CoCl_2$ **(K)** and at 39ₒC with $NiCl_2$ **(B)**, $ZnCl_2$ **(D)**, $FeCl_3$ **(F)**, $AlCl_3$ **(H)**, $MnCl_2$ **(J)**, or $Cocl_2$ **(L)** under a static condition. These symbols indicate the means of three replicates, and error bars indicate standard deviations: closed circle, control (0 mM), open circle, 0.01 mM; open diamond, 0.1 mM; and open square, 1.0 mM. Dotted and solid lines indicate the OD values of the first and second stages of cultivation, respectively.

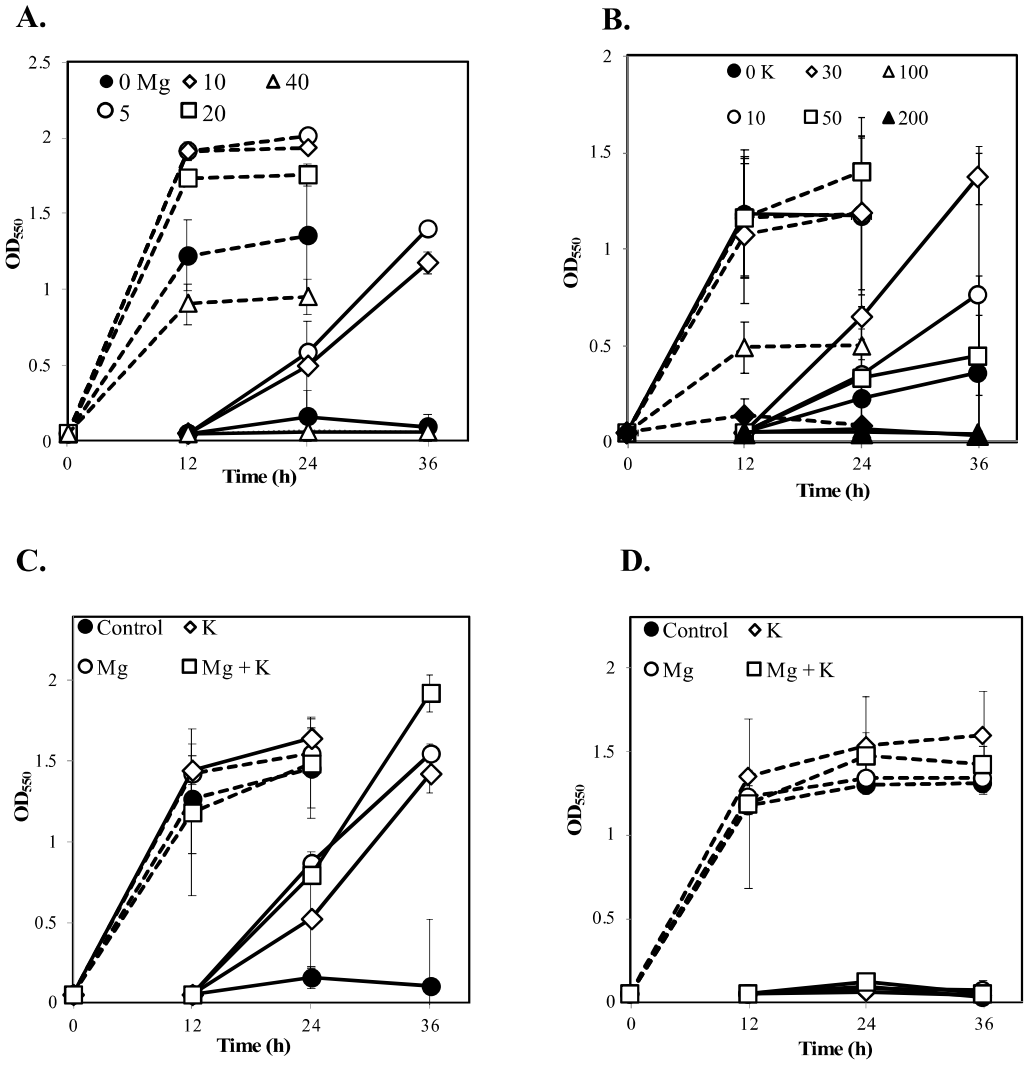
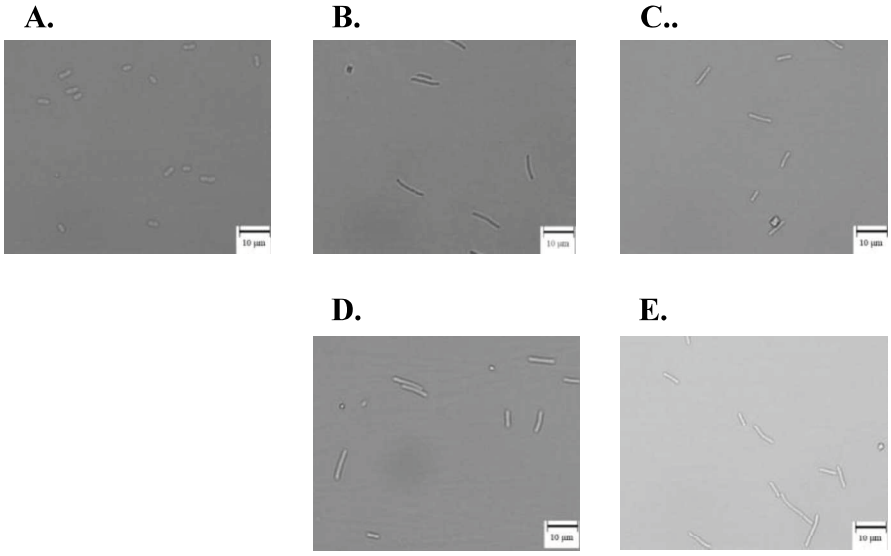


Figure 3.2 Effects of Mg^{2+} and K^{+} on the growth of *Zymomonas mobilis* TISTR 548 at critical high temperatures. Cells were cultivated in YPD medium with several concentrations of $MgCl_2$ (**A**: closed circle, 0 mM; open circle, 5 mM: open diamond, 10 mM; open square, 20 mM; and open triangle, 40 mM) and KCl (**B**: closed circle, control, 0 mM; open circle, 10 mM; open diamond, 20 mM; open square, 50 mM; open triangle up, 100 mM; and close triangle, 200 mM) at 39°C under static condition. At 39°C **(C)** and 39.5 °C **(D)**, 5 mM $MgCl_2$ (open circle), 30 mM KCl (open diamond), and a combination of these metals (open square), were added, and two-step cultivation was performed. Values and error bars represent means and standard deviations, respectively, for triplicate cultures.

3.4.2 Physiological effects of Mg and K on *Z. mobilis* TISTR 548 at CHT

Our previous report indicated that the cell length of *Z. mobilis* increased at CHT, and this increase reduced in thermotolerance- enhanced mutants (Kosaka *et al*., 2019). Indeed, cells grown at 39°C had longer cells than those grown at 30°C, which had granular shapes (**Figures 3.3 A, B**). Cell morphology observed following the addition of $MgCl_2$ or KCl indicated that cell length increased, with the increase in cell length being relatively lower following the addition of Mg^{2+} at 39°C than without the addition of metal ions (**Figure 3.3C**). On the other hand, the addition of KCl had no clear effect on cell length at 39°C, with a predominance of longer filamentous cells (**Figure 3.3D**). Cells cultured with both metals showed a mixture of granular and long filamentous shapes (**Figure 3.3E**). Indeed, the median value of measured cell length at 30°C, at 39°C, with $MgCl_2$ at 39°C, with KCl at 39°C, or with both metals at 39°C was 3.3, 7.6, 5.0, 7.2, or 6.1 μm, respectively (**Figure 3.3F**). Ethanol productivity at 39°C was also recovered to be close to the theoretical yield by adding $MgCl_2$ or KCl (**Figure 3.3G**). Accumulation of intracellular ROS was observed in *Z. mobilis* TISTR 548 at CHT (Kosaka *et al*., 2019). Addition of $MgCl_2$ or KCl considerably reduced intracellular ROS levels at 39°C, and the reduction strength was the highest for only K^+, followed by that for both metals and then only Mg^{2+} (**Figure 3.3H**).



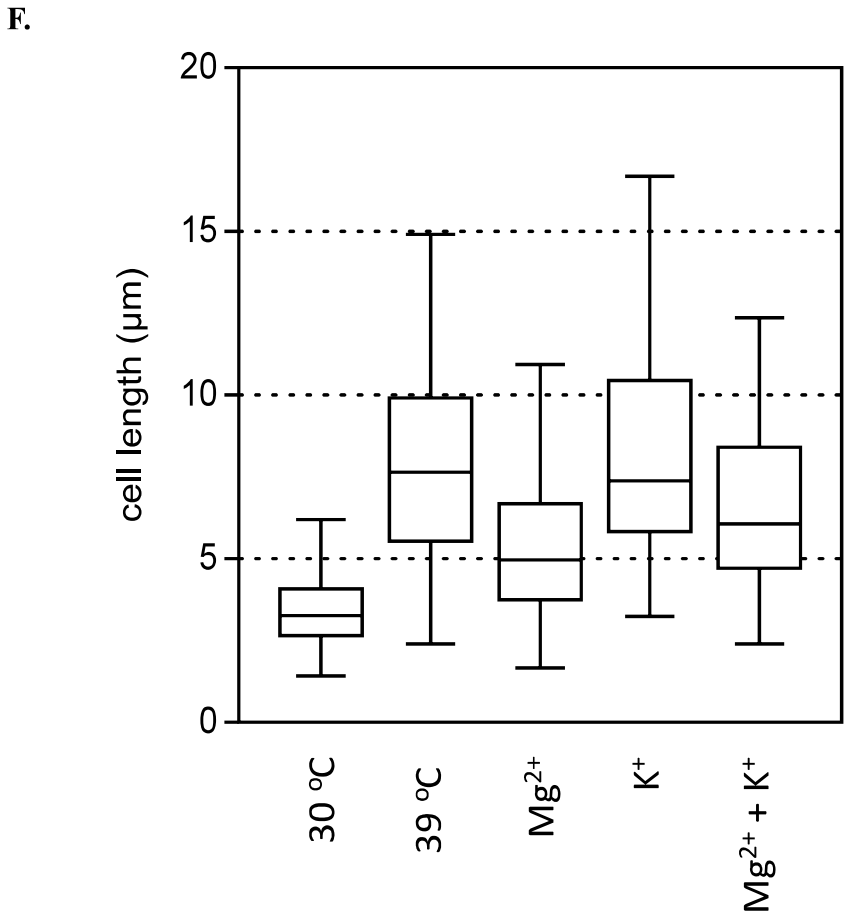


Figure 3. 3 Continued

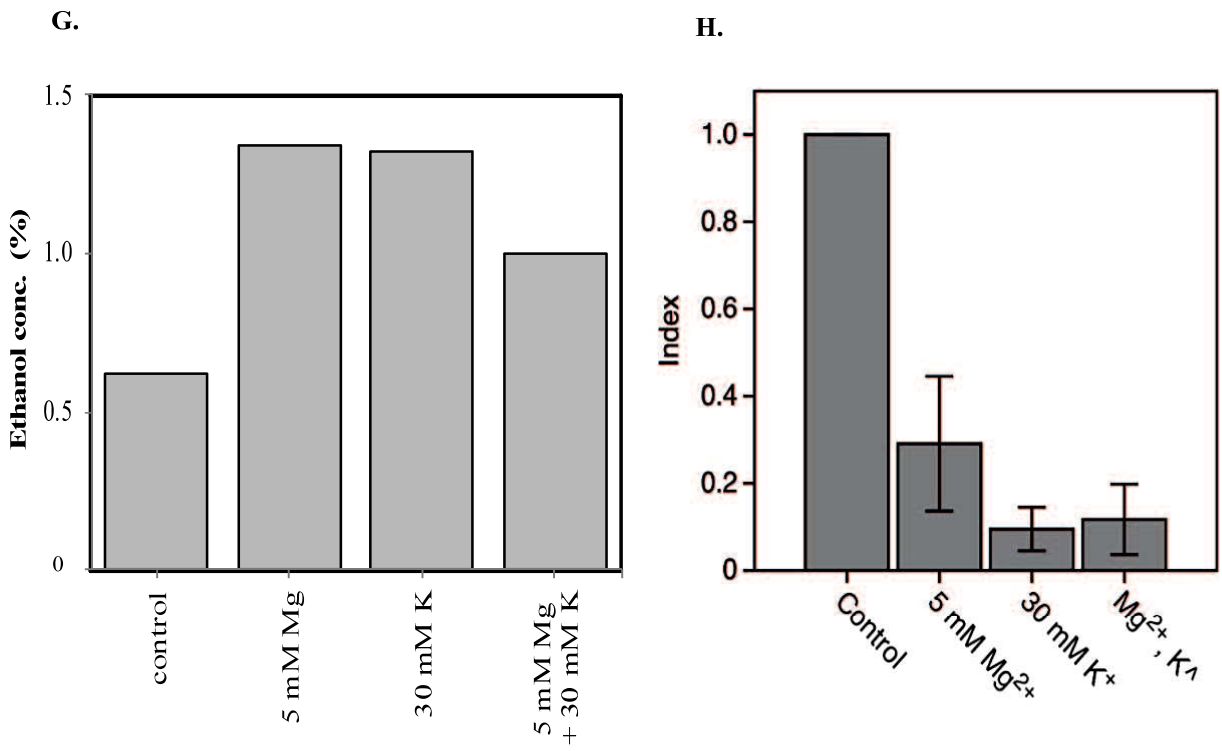


Figure 3. 3 Effect of Mg^{2+} and K^{+} on the physiology of *Zymomonas mobilis* TISTR 548 at critical high temperatures. Under all conditions, the final concentrations of the added $MgCl_2$ and KCl were 5 and 30 mM, respectively. **(A-E)** Morphology of cells grown in YPD medium at 30°C **(A)** or 39°C (B) with $MgCl_2$ (C), or KCl **(D)**, or both **(E)** for 7 h under a static condition. Photographs were taken at a magnification of x400. Bars indicate 10 µm. **(F)** A box plot of cell length from 100 cells measured in these conditions. The median cell length under each condition is shown as a number. **(G)** Ethanol concentration was measured in culture at 39°C for 12 h in YPD medium under a static condition. (H) The intracellular ROS level was measured in culture at 39°C for 12 h. Index was calculated as the ratio of the fluorescence intensity and protein concentration to the values of the control. Values and error bars represent means and standard deviation, respectively, for triplicate experiments.

3.4.3 Effects of Mg^{2+} and K^{+} on *Z. mobilis* TISTR 548 growth at CHT

Previous results indicated that Mg^{2+} and K^{+} somehow affect the cell physiology of *Z. mobilis* TISTR 548 at CHT and reduce intracellular ROS levels but probably by different mechanisms. Several bacteria use glutathione as a reducing agent to maintain a strongly reducing environment in cells, and glutathione peroxidase is an ROS-scavenging enzyme (Cabiscol Català *et al*., 2000). We observed the effect of glutathione with $MgCl_2$ or KCl on cell growth when glutathione was added at several concentrations: 4 mM glutathione inhibited cell growth at 39°C (data not shown) but 0.5 mM did not (**Figure 3.4A**). An Mg^{2+} plus glutathione effect was observed, but the effect was not considerably distinct from that observed following the addition of K^{+} or both metals (**Figures 3.4 B–D**). Next, an effective concentration of EDTA as a chelator of a divalent cation on cell growth at a CHT were explored, and then effects of metals under the presence of such a concentration of EDTA at a CHT were examined. When 0.05 mM EDTA was added to culture, cell growth was inhibited at 38°C (**Figure 3.5A**). $MgCl_2$ or KCl was subsequently added under the above condition, and the resulting effect was observed. The addition of Mg^{2+} rescued EDTA inhibition at CHT (**Figure 3.5B**), but that of K^{+} did not (**Figure 3.5C**).

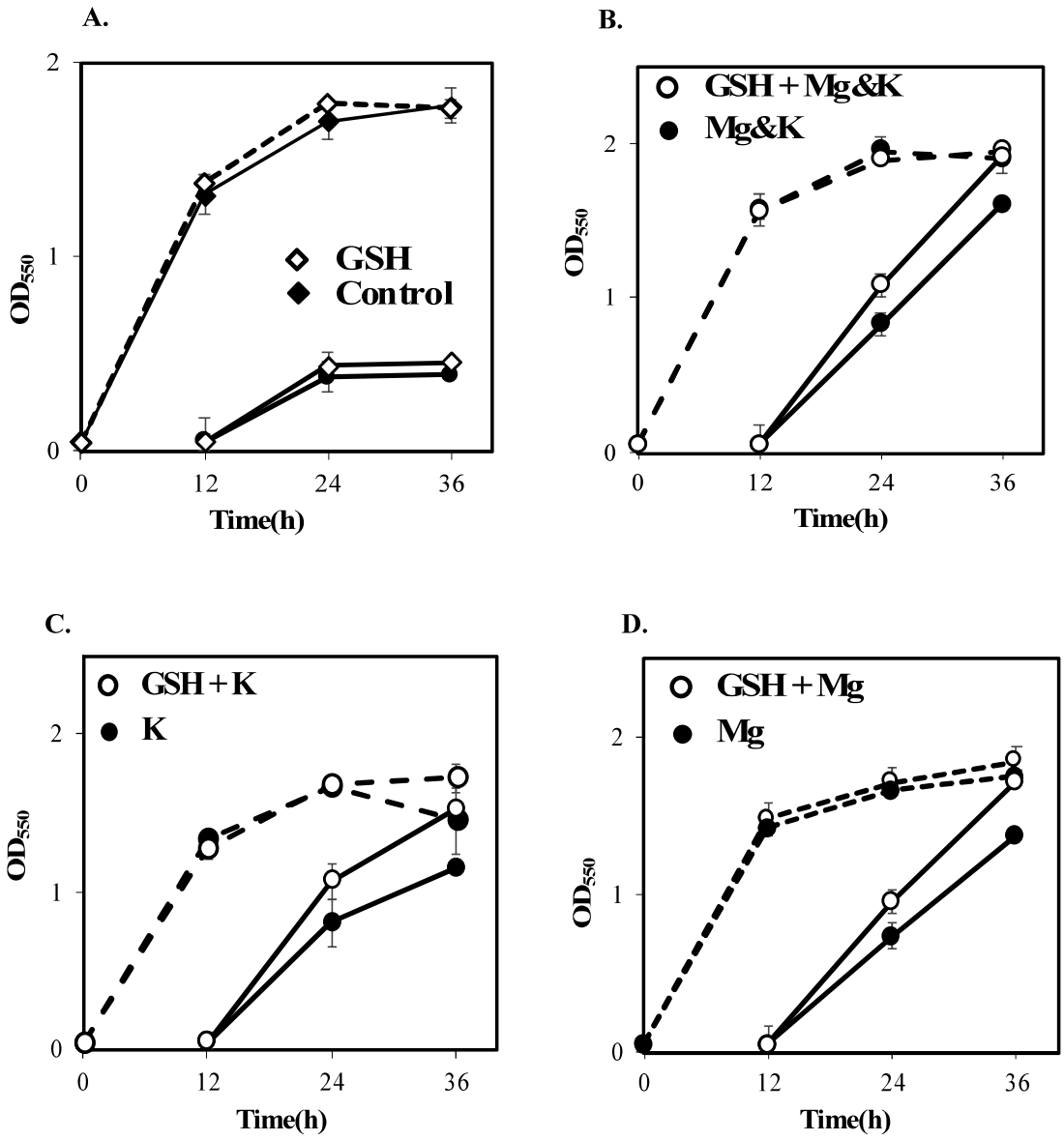


Figure 3.4 Effect of GSH with Mg^{2+} or K^{+} on the growth of *Zymomonas mobilis* 548. **(A)** Cells were grown in YPD medium with (open diamond) or without 0.5 mM GSH) control, closed diamond) at 39ₒC under a static condition. **(B-D)** Cells were grown in YPD medium containing 5 mM MgCl2 **(B)**, 30 mM KCl **(C)**, or both 5 mM $MgCl_2$ and 30 mM KCl **(D)** with (open circle) or without 0.5 mM GSH (closed circle) at 39ₒC under a static condition. After 12 h, the first-stage culture (dotted lines) was inoculated into a fresh medium and subjected to sybsequent (second stage) cultivation (solid lines) under the same medium condition. Additional and non-additional conditions of GSH are shown as open and closed circles, respectively. Values and error bars represent means and standard deviations, respectively, for triplicate cultures.

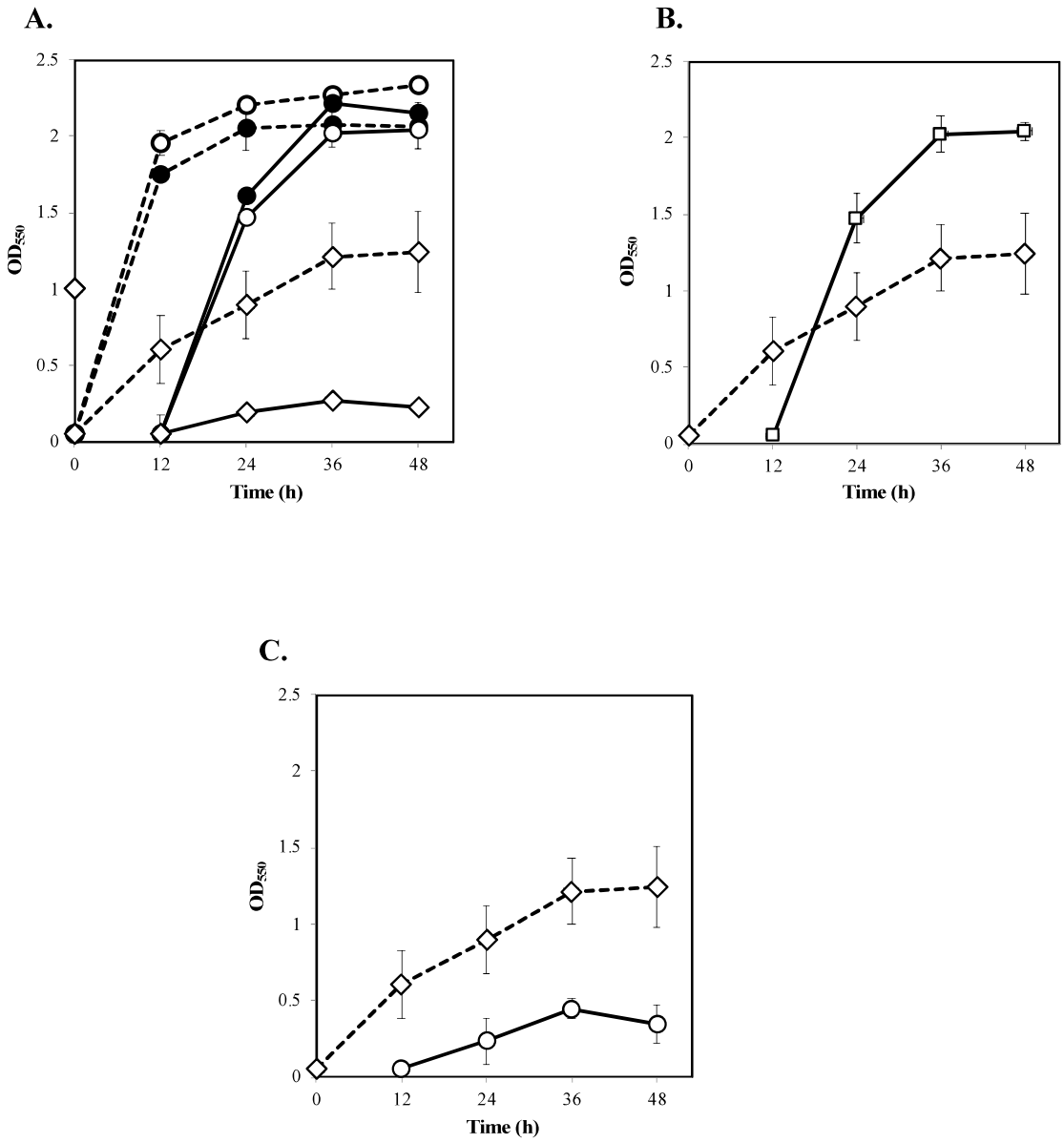


Figure 3.5 Effect of EDTA with Mg^{2+} or K^{+} on the growth of *Zymomonas mobilis* TISTR 548. **(A)** Cells were subjected to two-step cultivation (dotted line, first; solid line, second) in YPD medium with 0.01 mM EDTA (open circle), 0.05 mM EDTA (open diamond) or without EDTA (closed circle at 38^{o}C under a static condition. **(B,C)** At the first stage of cultivation, cells were cultured in YPD medium containing 0.05 mM EDTA (open diamond) at 38 ^{o}C, and after 12 h, at the second stage of cultivation (open circle), 5 mM $MgCl_2$ **(B)** or 30 mM KCl **(C)** was added to YPD medium containing 0,05 mM EDTA. Values and error bars represent means and standard deviations, respectively, for triplicate cultures.

3.4.4 Effects of Mg^{2+} and K^{+} on the Growth of Thermosensitive Mutants

In a previous study, 26 thermosensitive single gene-disrupted mutants of *Z. mobilis* TISTR 548 were isolated (Charoensuk *et al*., 2017). To observe the effects of the addition of Mg^{2+} and K^{+} on these thermosensitive mutants, their growth upon the addition of each metal was examined. Addition of Mg^{2+} significantly enhanced the growth of three mutants, whereas it inhibited the growth of one mutant (**Table 3.2**). Further, the addition of K^{+} enhanced the growth of nine mutants, i.e., almost one-third of all thermosensitive mutants, but it inhibited the growth of one mutant (C13-36) at 39.5°C (**Table 3.2**). Remarkably, the addition of both Mg^{2+} and K^{+} enhanced the growth of only one mutant with gene encoding for phospholipase D; they had the opposite effect on the mutant TC13 (**Table 3.2**). These results also suggest that Mg^{2+} and K^{+} affect the cell physiology of *Z. mobilis* TISTR 548 differently at CHT.

Table 3.2 Effects of Mg^{2+} and K^{+} on the growth of thermosensitive mutants from *Zymomonas mobilis* TISTR 548.

Group	Tn10-inserted gene[a]	Strain	Function	Protein type	Growth[b]			Effect of $MgCl_2$[c]	Effect of KCl[c]
					38 ˚C	39 ˚C	39.5 ˚C	Ratio (%)	Ratio (%)
WT (TISTR 548)					++++	+++++	+++	108 ± 10	105 ± 15
General metabolism	ZZ6_0707	TC01	Glucose sorbosone dehydrigenase	Soluble	+	+	-	114 ± 8	103 ± 23
	ZZ6_1376	TC03	5,10-methylenetetrahydrofolate reductase	Soluble	++++	++++	+	126 ± 1	118 ± 4
Membrane stabilization	ZZ6_1146	TE12	Glucosamine/fructose 6-phoshate aminotransferase	Membrane	+	+	-	119 ± 6	**328 ± 14**
	ZZ6_0929	3-24	Glycosyl transferase group 1	Soluble	+	-	-	138 ± 6	146 ± 2
	ZZ6_0923	TC04	Phospholipase D/Transphosphatidylase	Membrane	-	-	-	**176 ± 7**	**177 ± 10**
	ZZ6_1551	C12-36	Squalene hopene cyclase (Shc)	Soluble	-	-	-	131 ± 4	98 ± 13
	ZZ6_1046	C11-44	Tol/Pal system component TolQ	Membrane	+	+	-	157 ± 5	**519 ± 4**
	ZZ6_1043	1-2	Tol/Pal system component TolB	Soluble	+	+	+	115 ± 9	110 ± 5
	ZZ6_1254	C31-23	Protein export membrane protein SecD	Membrane	-	-	-	112 ± 5	**223 ± 7**
	ZZ6_1477	TC15	Preprotein translocase subunit Tim44	Membrane	-	-	-	151 ± 11	127 ± 2
	ZZ6_0158	TC14	Autotransporter secresion inner membrane protein TamB	Membrane	+	-	-	118 ± 9	165 ± 10

	ZZ6_1210	C13-36	Competence protein ComEC	Membrane	-	-	-	126 ± 7	33 ± 4
	ZZ6_0840	TC11	Hypothetical transmembrane protein	Membrane	-	-	-	**181 ± 8**	126 ± 4
	ZZ6_0541	TC09	Hypothetical transmembrane protein	Membrane	++++	+++	+	119 ± 1	116 ± 5
Transporter	ZZ6_1289	1-10	Putative Fe^{2+}/Mn^{2+} transporter	Membrane	-	-	-	**231 ± 3**	151 ± 4
DNA repair	ZZ6_0616	F32	DNA repair protein RadC	Soluble	++++	+++	+	113 ± 9	96 ± 4
	ZZ6_0934	C12-43	Exonuclease VII (XseA)	Soluble	-	-	-	156 ± 6	146 ± 1
	ZZ6_0681	TC10	DNA repair protein RadA	Soluble	+	+	-	101 ± 5	62 ± 8
tRNA/rRNA modification	ZZ6_0023	C12-44	tRNA/rRNA methyltransferase (SpoU)	Soluble	+++	++	++	117 ± 11	116 ± 12
Protein quality control	ZZ6_1659	C21-17	Zn-dependent peptidase	Soluble	++++	+++	++	99 ± 10	104 ± 16
	ZZ6_0980	TC05	Serine protease DegP	Soluble	-	-	-	153 ± 5	**172 ± 3**
Translational control	ZZ6_0702	TC12	ATP-dependent helicase HrpB	Soluble	-	-	-	98 ± 1	**212 ± 4**
Cell division	ZZ6_0979	TE19	ParA/MinD-like ATPase	Soluble	-	-	-	114 ± 8	90 ± 11
Transcriptional regulation	ZZ6_0019	C31-15	Trp repressor-binding protein WrbA	Soluble	-	-	-	137 ± 4	**577 ± 8**
Others	ZZ6_0962	C12-37	Pseudogene	Soluble	+	+	-	161 ± 9	121 ± 7
	ZZ6_0861	TC13	Hypothetical protein	Soluble	+	+	-	53 ± 3	327 ± 2

[a]Thirty-six thermotolerant genes were identified, and they were classified into 10 groups (Charoensuk *et al.*, 2017). [b]The growth of these mutants at 38, 39, and 39.5 ˚C compared with that of the parental strain on YPD medium. The symbols of "+" represent the degree of cell growth of mutants at a critical high temperature compared with that of the parental strain, whereas "-" indicates no growth. The ratio (%) was calculated from the OD550 values for cells grown at 39.5 ˚C for 24 h with 5 mM $MgCl_2$ or 30 mM KCl divided by those cells grown without metals. Values represent means ± standard deviations of three replicates. Bold values indicate a ratio of > 1.5 or < 0.5 against the parental strain.

3.5 Discussion

We observed the additional effects of Ni^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+}, Mn^{2+}, and Co^{2+} on *Z. mobilis* TISTR 548 growth at high temperatures, but these metals showed only negative effects (**Figure 1**). Among these, the effects of Fe^{3+} and Al^{3+} were negligible under the tested conditions (**Figures 1E–H**). However, 1.0 mM Ni^{2+}, Zn^{2+}, Mn^{2+}, and Co^{2+} clearly inhibited *Z. mobilis* TISTR 548 growth (**Figure 1**). A previous report indicated that the addition of 0.35 mM Zn^{2+} markedly inhibited ethanol productivity in *Z. mobilis* ZM4 probably by inhibiting metabolic enzymes (Liu *et al*., 2010). In the case of a different microorganism, i.e., *S. cerevisiae*, the quantity of Zn^{2+} , Mg^{2+} , and Mn^{2+} required for effective fermentation was 0.01, 0.05, and 0.04 g/L, respectively (Deesuth *et al*., 2012), implying that 0.1 mM metals are usually required for growth, but excess concentrations can inhibit growth. This implies that only specific metals enhance the growth of specific microorganisms.

Only Mg^{2+} and K^{+} enhanced *Z. mobilis* TISTR 548 growth and improved CHT from 38 to 39°C (**Figure 3.2A**). At CHT, the intracellular molecular components of bacterial cells leaked (Haight and Morita, 1966; Allwood and Russell, 1967). In *S. cerevisiae* and probably other microorganisms, the addition of K^{+} prevents ion leakage (Lam *et al*., 2014). K^{+} channels are activated when tension in the lipid bilayer is increased (Iwamoto and Oiki, 2018), and Mg^{2+} transporters are induced by heat treatment in *Salmonella enterica* (O'Connor *et al*., 2009). Regarding quantity, these two metal ions (K^{+} and Mg^{2+}) are the principal and second highest ions in bacterial cells found at concentrations of 100–500 mM (Ballal *et al*., 2007) and ~1 mM (Groisman et al., 2013), respectively. Therefore, the optimal concentrations of 30 mM K^{+} and 5 mM Mg^{2+} (**Figures 3.2 A, B**) are probably related to their intracellular concentrations, further suggesting that similar ion conditions enhance cell

metabolism by preventing ion leakage from cells or supporting ion transportation from the extracellular space. However, the effects of Mg^{2+} and K^{+} on the two common characteristics of bacteria, namely, cell elongation and ROS accumulation, observed in *Z. mobilis* TISTR 548 at CHT (Matsushita *et al*., 2016) were different. Cell length at CHT was suppressed by the addition of Mg^{2+} but not by that of K^{+} (**Figure 3.3F**). Although ROS accumulation reduced by the addition of both metals, the addition of K^{+} showed a stronger effect than that of Mg^{2+} (**Figure 3.3H**). The GSH results indicated that the additive effect of GSH was observed in both cases of Mg^{2+} and K^{+} (**Figures 3.4 B, C**), suggesting that the growth enhancement effect of Mg^{2+} or K^{+} does not arise directly from the action of GSH added exogenously. In a Gram-negative bacterium, *E. coli*, GSH is important for periplasmic redox homeostasis (Pittman *et al*., 2005) and heterogeneous expression of glutathione reductase allows the microbe to be hydrogen peroxide tolerance (Kim *et al*., 2009). It is assumed that, in *Z. mobilis* TISTR 548, GSH keeps periplasmic redox homeostasis and/or somehow makes cells tolerate oxidative stress by its reducing power, but the major effects at CHT by Mg^{2+} and K^{+} are not likely the action by GSH. Moreover, EDTA treatment showed that K^{+} did not complement the EDTA effect at CHT (**Figure 3.5C**). These results suggest that Mg^{2+} and K^{+} affect the cell physiology of *Z. mobilis* TISTR 548 at CHT using different mechanisms.

The effect of Mg^{2+} on the cell physiology of *Z. mobilis* TISTR 548 at CHT has been described: Mg^{2+} probably stabilizes membrane structure as proposed in *E. coli* (Charoensuk *et al*., 2017). Mg^{2+} stabilizes OM (Nikaido and Vaara, 1985), particularly LPS, where Mg^{2+} bridges lipid A (Nikaido, 2003). The present study results also showed that the addition of Mg^{2+} repressed cell elongation at CHT (**Figure 3.3F**) and restored the growth of the disrupted genes of *ZZ6_0923*, which encodes the cardiolipin biosynthesis protein (**Table 3.2**).

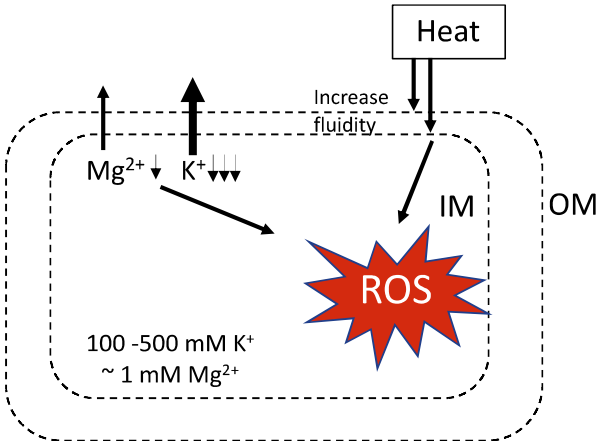
However, Mg^{2+} has been thought to stabilize proteins, enhance protein–nucleic acid interactions, mitigate oxidative stress, and act as a metabolic signal (O'Connor *et al*., 2009). Mg^{2+} is required to maintain cell metabolism, DNA replication, transcription and translation, and DNA stabilization (Xu *et al*., 2018), and it plays a role in enzyme activations. For instance, Mg^{2+} stabilizes pyruvate decarboxylase, an enzyme responsible for the decarboxylation of pyruvate in central metabolism, with thiamine diphosphate serving as a cofactor (Pohl *et al*., 1994). Besides, phosphoglycerate kinase uses Mg^{2+} as a cofactor (Andreini *et al*., 2008). Addition of Mg^{2+} reduced ROS accumulation at CHT (**Figure 3.3H**), and the growth of the disrupted Fe^{2+}/Mn^{2+} transporter (ZZ6_1289) recovered greatly (**Table 3.2**). Therefore, maintaining an intracellular Mg^{2+} concentration may enable heat tolerance either by ions or cytoplasmic Mg^{2+} sensors, proteins, and RNAs. (Groisman *et al*., 2013).

K^+, the most dominant intracellular cation, greatly contributes to pH homeostasis and turgor maintenance as well as bacterial osmotic adaptation, pH regulation, gene expression, and cell enzyme activation (Epstein, 2003; Ballal *et al*., 2007). Indeed, the addition of K^+ affected most cell physiology of *Z. mobilis* TISTR 548 at CHT (**Table 3.2**), e.g., the growth of 35% (9/26 strains) of mutants recovered greatly. These effects may contribute to reducing intracellular ROS levels (**Figure 3.3H**). Between these, K^+ probably facilitates the functioning of periplasmic proteins in *Z. mobilis* TISTR 548 due to the growth recovery of disrupted *tolQ*, *secD*, *tamB*, and *degP* (**Table 3.2**). The amount of intracellular K^+ directly affects membrane potential (Bakker and Mangerich, 1981), which is required for protein secretion to periplasm (Daniels *et al*., 1981). The membrane potential is hypothetically required for potassium transport from extracellular space to the cytoplasm via the membrane potential-driven K^+ uptake system (Kup, ZMO1209, and ZZ6_0125) in *Z.*

mobilis. Therefore, K^+ may also facilitate membrane potential maintenance in *Z. mobilis* TISTR 548.

Under CHT conditions (**Figure 3.6A**), the inner membrane fluidity increases to cause leakages of ions from cytoplasm and electrons from the respiratory chain, which lead to the accumulation of intracellular reactive oxygen species, resulting in damage of macromolecules of DNA, RNA, proteins and lipids, and thereby cells are elongated and unable to maintain intracellular homeostasis, causing cell death. However, by the addition of Mg^{2+} (**Figure 3.6B**), the OM is stabilized by binding of Mg^{2+} and the inner membrane is also stabilized, resulting in suppression of the leakage of intracellular ions as well as the leakage of electrons from the respiratory chain. On the other hand, by the addition of K^+ (**Figure 3.6C**), K^+ leakage is repressed to maintain homeostasis for cellular metabolism, by which intracellular ROS is reduced. Moreover, these observations suggest that Mg^{2+} and K^+ exhibit diverse, rather than single, effects on *Z. mobilis* TISTR 548. Interestingly, when both Mg^{2+} and K^+ exist in the medium at high concentrations, their crosstalk effects on cell physiology sometimes occur. These effects are partly specific to each ion; their additive effect on cell growth at CHT was observed, but that did not entail the whole sum of their effects (**Figure 3.2C**). The thermotolerance acquisition mechanisms of *Z. mobilis* upon the addition of Mg^{2+} and K^+ are more complex than the accumulated effects of their metals in accomplishing enhanced *Z. mobilis* growth at CHT.

A.



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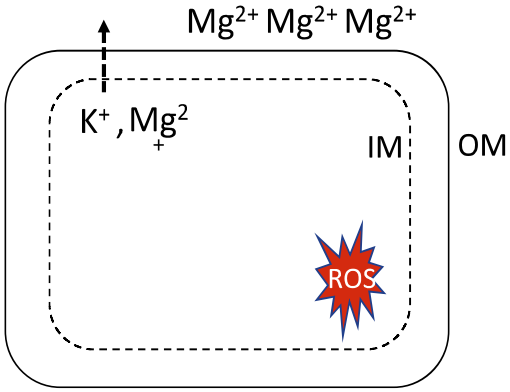

C.

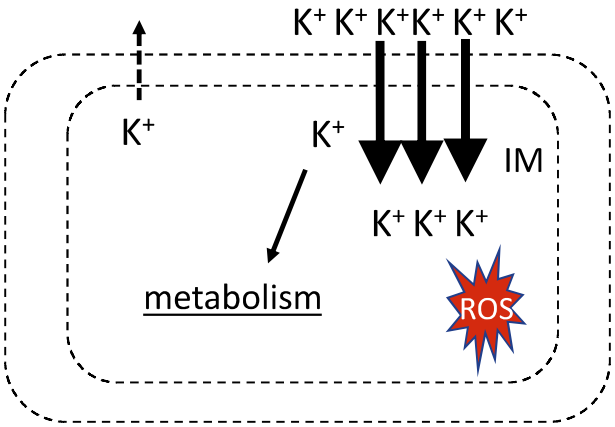

Figure 3.6 Models for the action mechanism of Mg^{2+} and K^{+} on *Z. mobilis* TISTR 548 at CHT. **(A)** Under CHT conditions, **(B)** by the addition of Mg^{2+}, **(C)** by the addition of K^{+}. IM, inner membrane; OM, outer membrane; ROS, reactive oxygen species.

3.6 Conclusion

Among various metals, only Mg^{2+} and K^{+} enhanced the thermotolerance of *Z. mobilis* TISTR 548. The primary effects of Mg^{2+} and K^{+} on the cell physiology of *Z. mobilis* TISTR 548 are largely different, but these metals reduce intracellular ROS accumulation. Based on the study results, several strategies for improving the CHT of *Z. mobilis* by membrane stabilization and intracellular metabolism maintenance can be expected. Further research is needed to reveal these mechanisms for improving its growth at CHT.

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APPENDIX

A

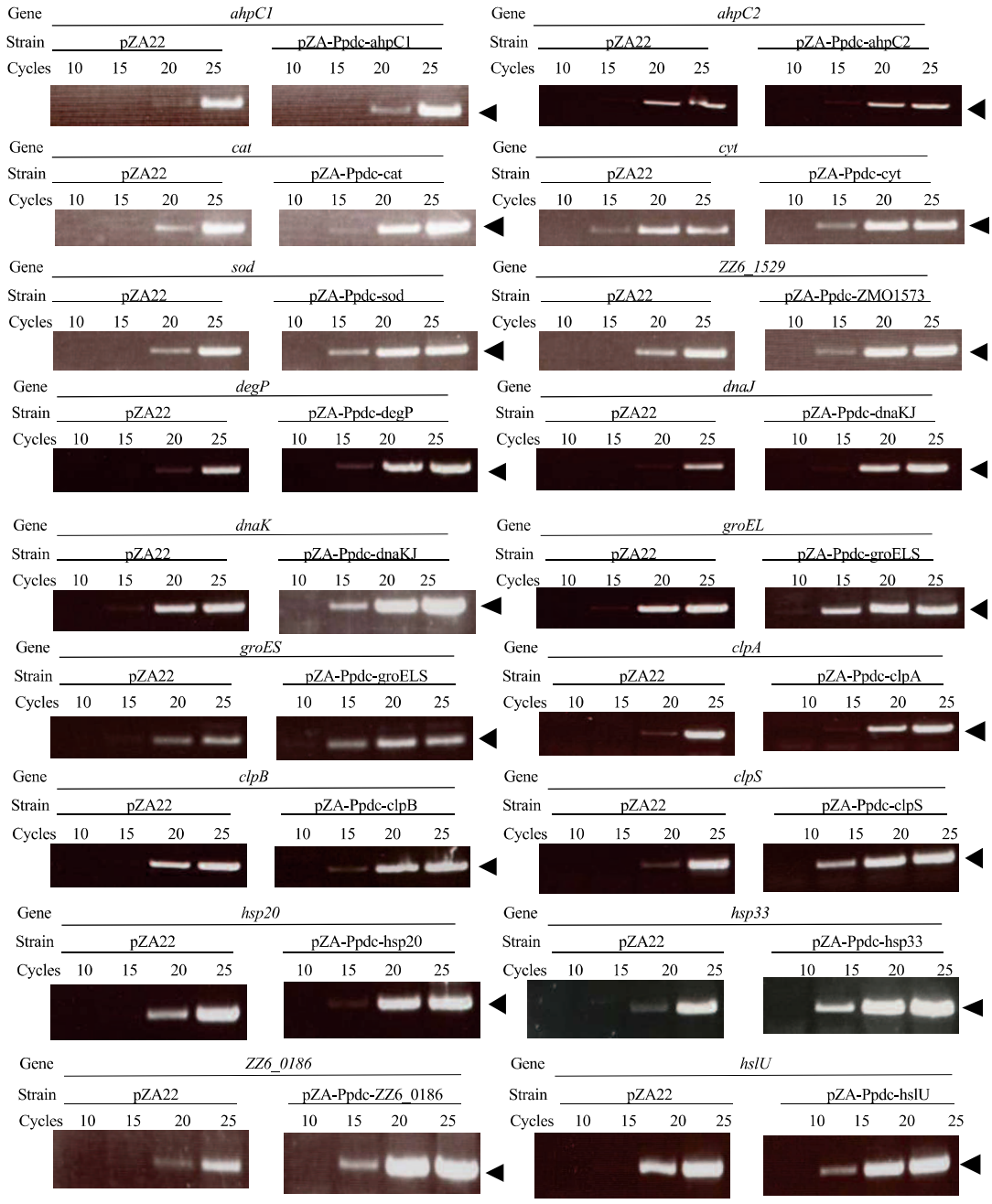

Figure S1. Continued

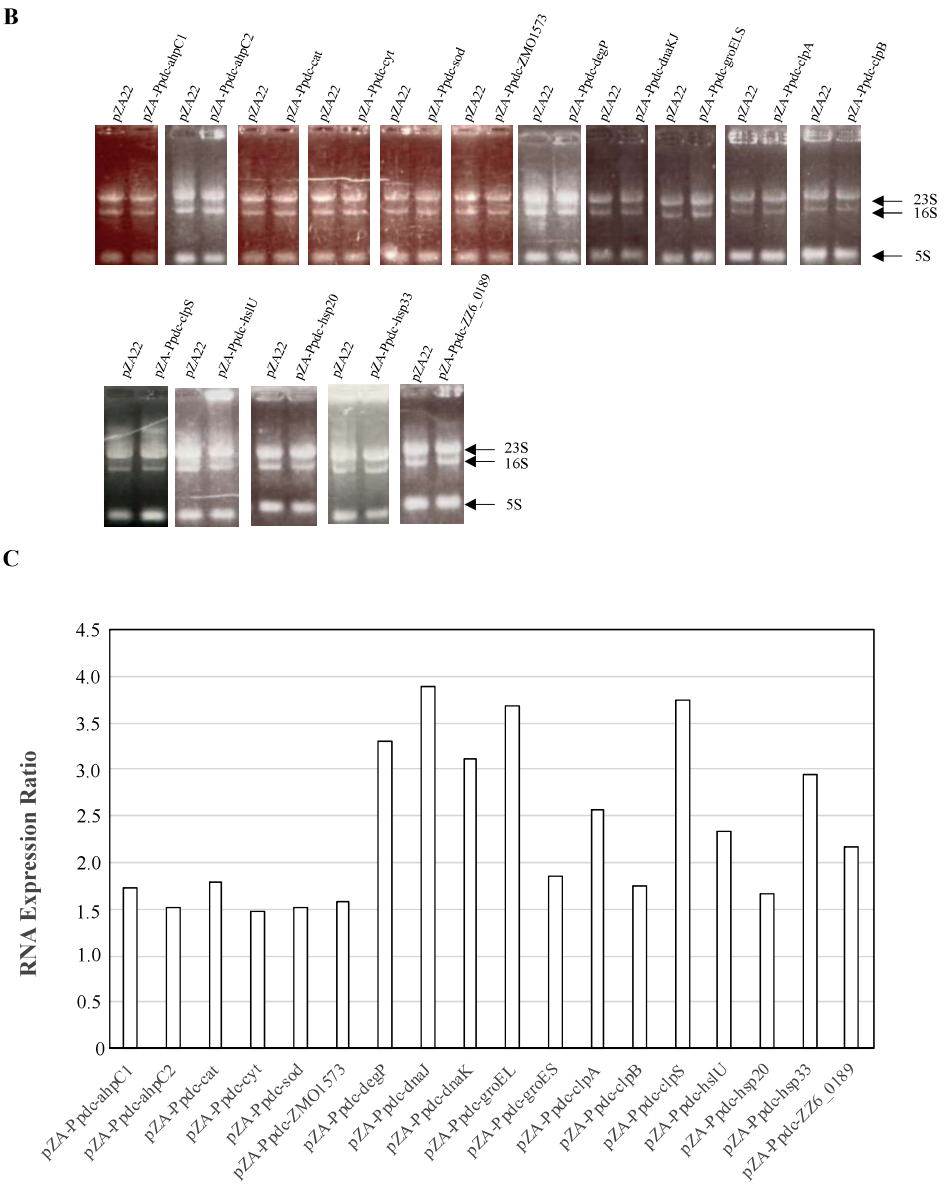

Figure S1. Checking of increased expression of genes for RSEs and HSPs from recombinant plasmids. Strains are shown by introduced gene names. Total RNA was prepared from cells cultured at 30°C as described in Materials and Methods. RT-PCR was performed with primers specific for each gene to amplify approximately 500-bp DNA fragments. After RT reaction, PCR was performed for 10, 15, 20, and 25 cycles and the products were analyzed. Each PCR product and total RNA (10 μg) as a control were subjected to 1.2% agarose gel electrophoresis and staining with ethidium bromide **(A, B)**. Intensity of stained bands was determined by using ImageJ, and the ratio of the intensity of bands from cells harboring a recombinant plasmid to that from cells harboring an empty vector, pZA22, was calculated. White columns represent the relative intensities as an expression ratio (%) **(C)**.

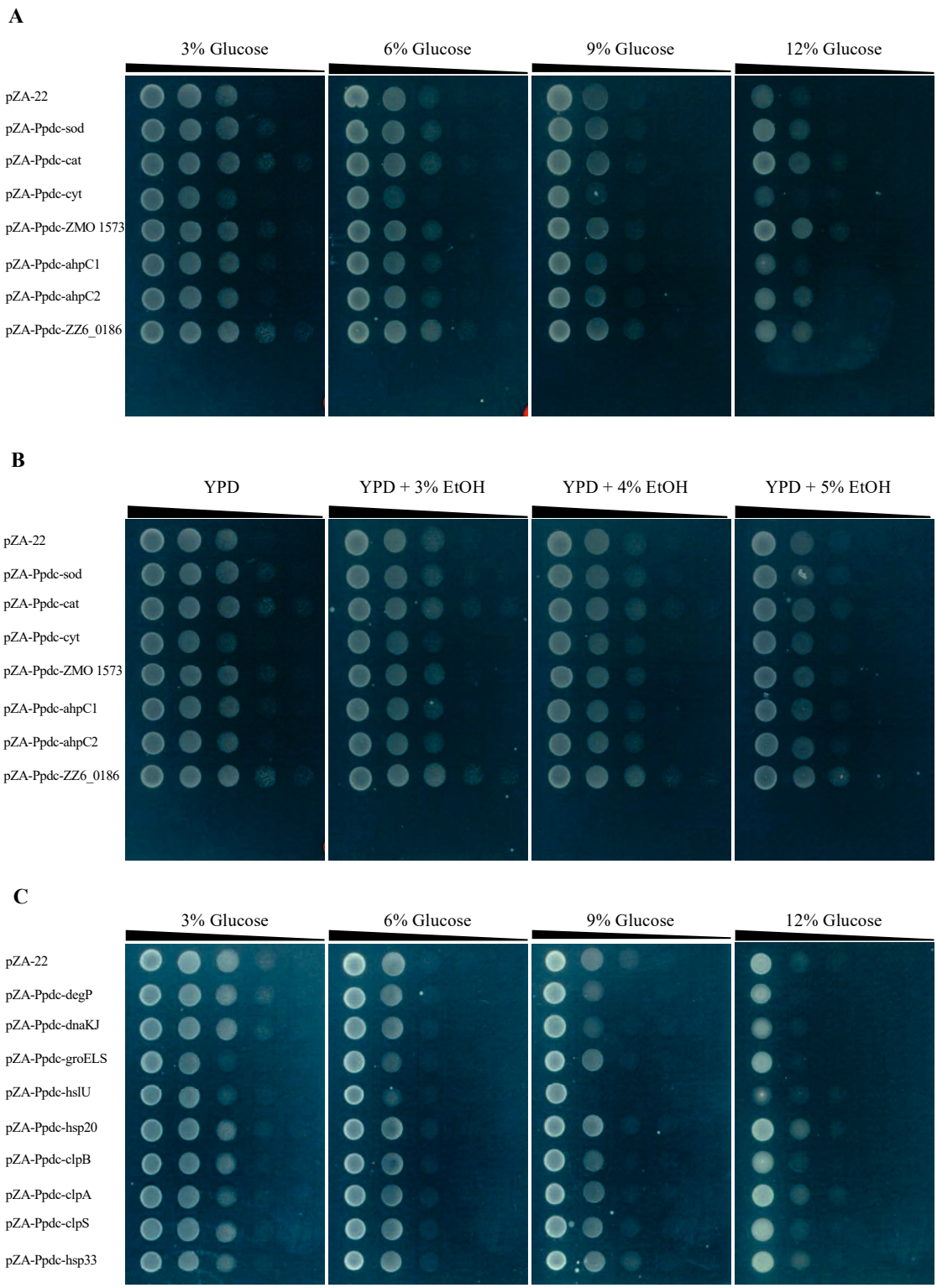

Figure S2. Continued

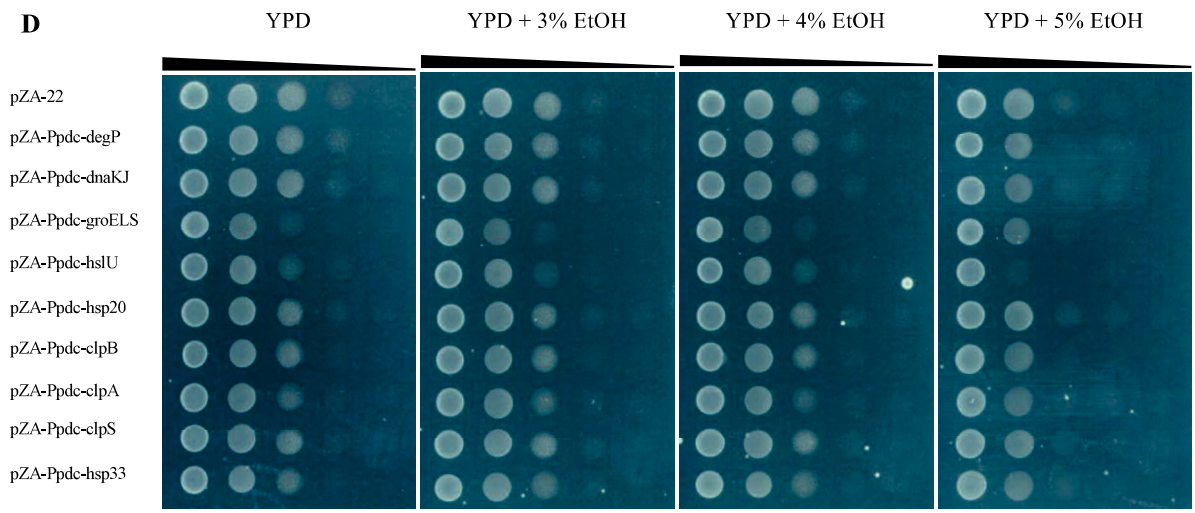

Figure S2. Effects of increased expression of genes for RSEs **(A,B)** and HSPs **(C,D)** on tolerance to various concentrations of glucose and ethanol. Cells were grown at 30°C in YPD medium containing chloramphenicol overnight. The cell culture at OD_{550} of about 1.0 was serially diluted (10_0–10_4-times dilution) and spotted on YPD agar plates containing 3–12% glucose or 3–5% ethanol, and incubated at 30°C for 48 h.

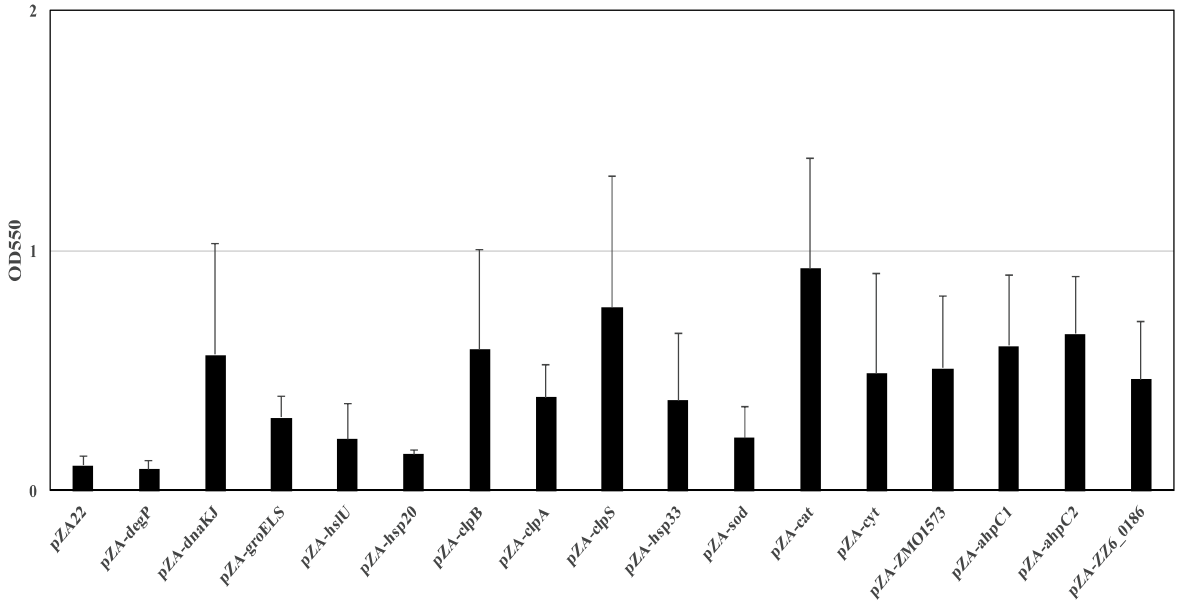

Figure S3. Effects of increased expression of genes for RSEs **(A)** and HSPs **(B)** on tolerance to H_2O_2. Cells were grown at 30°C in YPD medium containing 0.1 mM H_2O_2, and optical density at OD_{550} of the culture was measured at 12 h.

Table S1. Plasmid used in this study

Plasmid	Description	Reference and source
pZA22	Cml^r	Misawa *et al*. (1986)
pZA-Ppdc-ahpC1	pZA22 with the fragment fused *pdc* promoter and *ahpC 1* gene by In-fusion cloning	This study
pZA-Ppdc-ahpC2	pZA22 with the fragment fused *pdc* promoter and ahpC2 gene by In-fusion cloning	This study
pZA-Ppdc-cat	pZA22 with the fragment fused *pdc* promoter and *cat* gene by In-fusion cloning	This study
pZA-Ppdc-clpA	pZA22 with the fragment fused *pdc* promoter and *clpA* gene by In-fusion cloning	This study
pZA-Ppdc-clpB	pZA22 with the fragment fused *pdc* promoter and *clpB* gene by In-fusion cloning	This study
pZA-Ppdc-clpS	pZA22 with the fragment fused *pdc* promoter and *clpS* gene by In-fusion cloning	This study
pZA-Ppdc-cyt	pZA22 with the fragment fused *pdc* promoter and *cyt* gene by In-fusion cloning	This study
pZA-Ppdc-degP	pZA22 with the fragment fused *pdc* promoter and *degP* gene by In-fusion cloning	This study
pZA-Ppdc-dnaKJ	pZA22 with the fragment fused *pdc* promoter and *dnaKJ* gene by In-fusion cloning	This study
pZA-Ppdc-groELS	pZA22 with the fragment fused *pdc* promoter and *groELS* gene by In-fusion cloning	This study
pZA-Ppdc-Hsp20	pZA22 with the fragment fused *pdc* promoter and *ibpA* gene by In-fusion cloning	This study
pZA-Ppdc-Hsp33	pZA22 with the fragment fused *pdc* promoter and *hsp33* gene by In-fusion cloning	This study
pZA-Ppdc-hslU	pZA22 with the fragment fused *pdc* promoter and *hslU* gene by In-fusion cloning	This study
pZA-Ppdc-sod	pZA22 with the fragment fused *pdc* promoter and *sod* gene by In-fusion cloning	This study
pZA-Ppdc-ZMO1573	pZA22 with the fragment fused *pdc* promoter and *ZZ6_1529* gene by In-fusion cloning	This study
pZA-Ppdc-ZZ6_0186	pZA22 with the fragment fused *pdc* promoter and *ZZ6_0186* gene by In-fusion cloning	This study

Table S2. Primers used in this study

Name	Sequence (5’ → 3’)
pZA-5’	gatcctctacgccggacgcatcgtg
pZA-3’	gatccacaggacgggtgtggtcgcc
Ppdc-5’	cggtacccggggatcacgggctatccttcaaaagaagaatc
Ppdc-3	tgcttactccatatattcaaaacac
pZA-5’	atggaagccggcggcacctc
pZA-Ppdc-3'	tgcttactccatatattcaa
ahpC1-5’	tatatggagtaagcaatggcttttgaaaaaataaaaaaaa
ahpC-1-3’	cgactctagaggatcctatcgacggtttgatttctgccagt
ahpC2-5’	tatatggagtaagcaatgcttactgttggtgataa
ahpC2-3’	gccgccggcttccatttaggctttaggatcaagca
cat-5’	tatatggagtaagcaatgactagacccaatcttcagactg
cat-3’	cgactctagaggatcgcagcctattttataacaggctatc
clpA-5’	tatatggagtaagcaatgccatcttttgccaaagc
clpA-3’	gccgccggcttccattcaggattcatccagtgttt
clpB-5’	tatatggagtaagcaatgaattttgagaaattaac
clpB-3’	gccgccggcttccatttaggctgacactgaatctt
clpS-5’	tatatggagtaagcaatgagcggcgacaaggattt
clpS-3’	gccgccggcttccatttaggctttttctaaagtgc
cyt-5’	tatatggagtaagcaatgaatatcaaggctttattcgggt
cyt-3’	cgactctagaggatcaggaggatgattatttagaaggcaa
degP-5’	tatatggagtaagcagtgcgctacgcctatgccgt
degP-3’	gccgccggcttccatttatttgtttttagtcggag
dnaKJ-5’	tatatggagtaagcaatgggtaaagttataggtat
dnaKJ-3’	gccgccggcttccatttgcctgaaatagctttcta
groESL-5’	tatatggagtaagcaatgggggacattggcacttc
groESL-3’	gccgccggcttccattcaacccatcagaagataca
hslU-5’	tatatggagtaagcaatgactgatgcccttacgcc
hslU-3’	gccgccggcttccatttagagaatatatttactca
hsp33-5’	tatatggagtaagcaatgagtgagaatatccagaa
hsp33-3’	tatatggagtaagcaatgagtgagaatatccagaa
ibpA-5’	tatatggagtaagcaatgcgtgcttttgatcttac
ibpA-3’	gccgccggcttccatctaggcggcttgctgcttta
sod-5’	tatatggagtaagcaatggcttttgcattacccgccctgc
sod-3’	gccgccggcttccatgcttttggtcttaagcctgttttaa
ZMO1573-5’	atatggagtaagcagtgtccgaacccctgcccca
ZMO1573-3’	gccgccggcttccatttattcaacctcatccaaaaagt
ZZ6_0186-5’	tatatggagtaagcaatgagtgctgaatcctattc
ZZ6_0186-3’	gccgccggcttccattcaatcctccttgaaatcaa

Table S3. Primers used for RT-PCR primers in this study

Name	Sequence (5’ → 3’)
ahpC1-5'	AGCATCTCATTTAGGCGCGA
ahpC1-3'	TGTATCGCTTCCATCGCCTT
ahpC2-5'	AGCAGGGTGTATCTGCCTTG
ahpC2-3'	TTCGCCTTTATGCCAGTTGC
cat-5'	TCAGGATGTTGAGCAAGCCG
cat-3'	CCAAGATCTGCATCGCAACG
clpA-5'	TGTCCAGTGGGACTATCCGT
clpA-3'	AACCGGCACGGCTTAATTTG
clpB-5'	ACCAGAGAATTGCGCCTGAA
clpB-3'	GCGGCGGATTTCTTCATCAC
clpP-5'	TGTAACCGGTCAGGTCGAAG
clpP-3'	TCTTCAGAAGCAACCGGACG
clpS-5'	AGCGGCGACAAGGATTTTGA
clpS-3'	ACGGATGTTGATGCTGACGG
cyt-5'	ATACAGCCTATTCCGGCAGC
cyt-3'	CCGCGTTTTTCCTGTTCGTT
degP-5'	GCGCTGTTGTCGAATCCATC
degP-3'	CTGTCAGCACCTGTATGGCA
dnaK-5'	CACCTTCCCAGATTTCGGCT
dnaK-3'	TTGACTTCGGTCGTCTGAGC
dnaJ-5'	CCTCTGTCTGTGACGCTTGT
dnaJ-3'	CGCGCCGTTTCAGTTGTTTA
groEL-5'	GATGGATCTGAAGCGCGGTA
groEL-3'	CACCAAAGCCAGGAGCCTTA
groES-5'	TCGTCCGCTACATGATCGAG
groES-3'	AACTTCGGTGCCTGACCATT
hslU-5'	GCATGGCGTCGTTTGATTGA
hslU-3'	CAGCTGGCGGATACCATCTT
hsp33-5'	TGGTGCGTCTGAATAGCGTT
hsp33-3'	ATATCCATCCGCGGCCATTT
ibpA-5'	CCCTTTCTTGCGTTCGTCTG
ibpA-3'	TAGGCGGCTTGCTGCTTTAG
sod-5'	AGAAACTGGCTGGTCTGAGC
sod-3'	CTTTGGCATATTTCGGGCGG
ZMO1573-5'	CTTTGCGTCGGACAGTTTCG
ZMO1573-3'	CCGGCTTTGTTTCGTCATCC
ZZ6_0186-5'	TGCGCTACCTGTGATGGTTT
ZZ6_0186-3'	TGGCGGTAATGCTTGTCCAT

ACKNOWLEDGEMENT

First of all, I would like to express my sincere gratitude to my advisor Prof. Mamoru Yamada for the continuous support of my Ph.D study and research, for his patience, motivation, enthusiasm, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis. Without his guidance and constant feedback, this Ph.D would not have been achievable. Beside my advisor, I would like to thank to Assoc. Prof. Tomoyuki Kosaka as my co-advisor, who also support my study by provided me his time for valuable discussion and helped at many works, especially also for giving an opportunity to join with his team and make the second publication was possible.

My sincere thanks also go to Prof. Kazunobu Matsushita, Prof. Toshiharu Yakushi and Asst. Prof. Naoya Kataoka, for their insightful comments and encouragement, also for the hard questions sometimes which incented me to widen my research from various perspectives. My thanks also go out to Dr. Masayuki Murata, Dr. Suprayogi, Dr. Mochamad Nurcholis, and Dr. Kaewta Sootsuwan for their fatefully helps, comments, discussions and suggestions during my research or other work performance. My deep appreciation also goes to fellow labmates for their help in numerous ways and also friendship and warm memories. Especially the *Zymomonas* team members, their excellent work during data collection has made an invaluable contribution towards my Ph.D.

I also gratefully acknowledge the funding received towards my PhD from the BUDI-LN LPDP, the scholarship program, collaborated by Ministry of Research, Technology, and Higher Education of Indonesia and Ministry of Finance of Indonesia. I also thanks to

Brawijaya University for provision of many supporting parts during my study abroad and Yamaguchi University for providing many facilities during my Ph.D.

Last but not least, I would like to thank to my family: my mothers with the power of her prayer, who always believing in me and encouraging me to follow my dreams, my sisters Prima and Putri for their supports, and also my husband Agni Prajna Yadi, who has been by my side trough out this PhD, thank you for love and support.

Thank you.

Sakunda Anggarini

Ph.D Student of Graduate School of Science and Technology for Inovation
Yamaguchi University
September 2020

LIST OF PUBLICATIONS

1. **Improvement of Thermotolerance of *Zymomonas mobilis* by Genes for Reactive Oxygen Species-Scavenging Enzymes and Heat Shock Proteins.**

   Sakunda Anggarini, Masayuki Murata, Keisuke Kido, Tomoyaki Kosaka, Kaewta Sootsuwan, Pornthap Thanonkeo, and Mamoru Yamada

   Frontier in Microbiology, 2020, 10 / 3073 : 1-14, doi: 10.3389/fmicrb.2019.03073

2. **Enhancement of Thermal Resistance by Metal Ions in Thermotolerant *Zymomonas mobilis* TISTR 548.**

   Tomoyuki Kosaka, Aya Nishioka, Tomoko Sakurada, Kento Miura, Sakunda Anggarini, and Mamoru Yamada

   Frontier in Microbiology, 2020, 11 / 502 : 1-10, doi:10.3389/fmicb.2020.00502