# Molecular mechanism for coping with critical heat and oxidative stresses in thermotolerant *Zymomonas mobilis*

(耐熱性 *Zymomonas mobilis* における限界熱ストレスと
酸化ストレスに対する対処分子)

**Kannikar Charoensuk**

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# List of abbreviations

| | |
|---|---|
| bp | Base pair |
| BSA | Bovine serum albumin |
| °C | Degree Celsius |
| DNA | Deoxyribinecleic acid |
| et al. | and others |
| g | gram (s) |
| h | hour (s) |
| kb | kilobase (s) |
| kDa | kilodalton (s) |
| Km | Kanamycin |
| L | Liter (s) |
| LB | Luria-Bertani medium |
| M | Molar |
| mg | milligram (s) |
| min | minute (s) |
| mM | millimolar |
| µg | microgram (s) |
| µl | microliter (s) |
| µM | micromolar (s) |

# List of abbreviations (cont.)

| | |
|---|---|
| NAD+ | oxidized nicotnamide adenine dinecleotide |
| NADP+ | oxidized nicotnamide adenine dinecleotide phosphate |
| NADH | reduceded nicotinamide adenine dinecleotide |
| NADPH | reduceded nicotinamide adenine dinecleotide phosphate |
| nm | nanometer (s) |
| PCR | Plymerase chain reaction |
| rpm | revolutions per minute |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | Sodium dodecyl sulfate polyacrylamide gel electrophresis |
| YPD | Yeast Peptone Dextrose |
| % | percent |

# Chapter 1

## Introduction and review of literatures

### 1.1 General introduction

The world population on Earth of July 2008 is estimated to be just over 6.68 billion expected to reach nearly 9 billion by the year 2042. The additional people will be almost all in poorer developing countries. A rapidly growing world population required more food (http://www.commondreams.org/news2000/0523-01.htm). Global demand for cereals is projected to increase by 40 %. Meat demand is expected to increase by 58 % and demand for roots and tubers by 37 %. The most of this increase coming from the developing world. Demand for fruits, vegetables and seasonings as well as nonfood farm products will also rise. But future increases in food production and new land to expand the agricultural base are likely to be more difficult because a complex range of environmental and social factors.

In too many foods production regions of the world have severely depleted because supplies of oil and gas are essential to modern agriculture techniques, a fall in global oil supplies could cause spiking food prices and unprecedented famine in the coming decades. Nowadays crude oil prices behave much as any other commodity with wide price swings in times of shortage or oversupply. The crude oil price cycle may extend over several years responding to changes in demand as well as OPEC and non-OPEC supply. In 1983 crude oil price was about 30 US. Dollar per barrel during 25 years the price raise dramatically with 400 % at 140 US. per barrel. Because of world high dependence of most modern industrial transport, agricultural and industrial systems on the relative low cost and high availability of oil will cause the oil-peak production decline and possible severe continue increases in the price of oil to have negative implications for the global economy (http://en.wikipedia.org/wiki/Peak_oil). The alternative energy comes to substitute for oil. In 2006, about 18% of global final energy consumption came from renewable source, with 13% coming from traditional biomass. Hydropower was the next largest renewable source, providing 3%, followed by hot water/heating, which contributed 1.3%. Modern technologies, such as

geothermal, wind, solar, and ocean energy together provided some 0.8% of final energy consumption (http://www.i-sis.or g.uk/SustainableWorldInitiativeF.php).

Bio-fuel as a member of renewable energy which uses the energy contained in organic matter - crops such as sugarcane or corn include wheat crops, waste straw, willow and popular trees, sawdust, reed canary grass, cord grasses, Jerusalem artichoke, myscanthus and sorghum plants. To produce ethanol, an alternative to fossil-based fuels like petrol.

Ethanol or ethyl alcohol ($C_2H_5OH$) is a clear colorless liquid; it is biodegradable, low in toxicity and causes little environmental pollution if spilt. Ethanol burns to produce carbon dioxide and water. Ethanol is a high octane fuel and has replaced lead as an octane enhancer in petrol. By blending ethanol with gasoline and oxygenate the fuel mixture so it burns more completely and reduces polluting emissions. Ethanol fuel blends are widely sold in the United States and still increase around the world (http://www.esru.strath.ac.uk/EandE/Web_sites/02-03/biofuels/what_bioethanol.htm). The production of bioethanol around the world was 41 billion liters in 2004. The largest producers in the world are Brazil with 37%, US with 33% and Asia with 14%. Below is a description of EU's focus on bioethanol (http://www.biogasol.dk/3me4.htm). Global demand to approach 90 million metric tons in 2011 - Demand for biofuels will expand almost 20 % per year through 2011 to 92 million metric tons. On another hand the World Bank reported cause of increased bio fuel production has contributed to the rise in food price. So increase the efficiency of bioethanol production processes such as used the ethanologenic thermotolerant microorganism is expected to become one of the economical next-generation fermentation technologies.

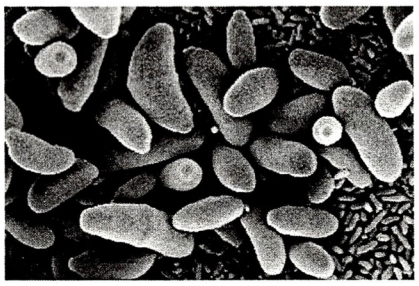
## 1.2 Microorganism genetic developments for bioethanol production

The importance of yeast cell physiology in alcohol fermentations has been emphasized by several researches such as for fermentations of pentose by *S. cerevisiae* [Kötter et al., 1990; Tantirungkij et al., 1993] and *Z. mobilis* [Deanda et al., 1995], for fermentations of high-gravity cereal [Thomas and Ingledew, 1992] and for fermentations of sugar cane molasses [Walker *et al.*, 1996]. Genetic engineering micro-organisms for utilization of useful materials for ethanol production are noted in Table 1.1.

**Table 1.1** Genetic engineered microorganism for utilization of useful materials for ethanol production.

| Microorganisms | Gene engineering | Useful materials | Reference |
|---|---|---|---|
| *Escherichia coli* | Alcohol dehydrogenase II and pyruvate decarboxylase from *Zymomonas mobilis* | Glucose | Ingram LO *et al.*, (1987), Ohta K *et al.*, (1991) |
| *Klebsiella oxytoca* | Plasmids with a-Amylase *Bacillus stearothermophilus* and the pullulanase gene from *Thermoanaerobium brockii* | Starch and maltose | Vera Lu´ cia dos Santos *et al.*, (1999) |
| *K. oxytoca* | Pyruvate decarboxylase and alcohol dehydrogenase from *Z. mobilis*; and endoglucanase from *Erwinia chrysanthemi* | Cellulose | Zhou S *et al.*, (2001), Zhou S and Ingram LO (2004) |
| *Z. mobilis* | xylose isomerase, xylulokinase, L-arabinose isomerase, L-ribulokinase, L-ribulose 5-phosphate 4-epimerase, transaldolase and transketolase from *E.coli* | Xylose, arabinose that produce by hydrolysis of hemicellulose and cellulose or starch | Deanda K *et al.*, (1995) |
| *Zymobacter palmae* | Xylose isomerase, xylulokinase, transaldolase and transketolase from *E.coli* | Xylose | Yanase H *et al.*, (2007) |
| *Saccharomyces cerevisiae* | Xylose reductase, xylitol dehydrogenase from *Pichai stipitis* | xylose | Kötter *et al.*, (1990), Tantirungkij, M *et al.*, (1993) |

## 1.3 The ethanologenic bacterium *Z. mobilis* and its biotechnological potential



*Bacteria*;
*Proteobacteria*;
*Alphaproteobacteria*;
*Sphingomonadales*;
*Sphingomonadaceae*;
*Zymomonas*;
*Zymomonas mobilis*

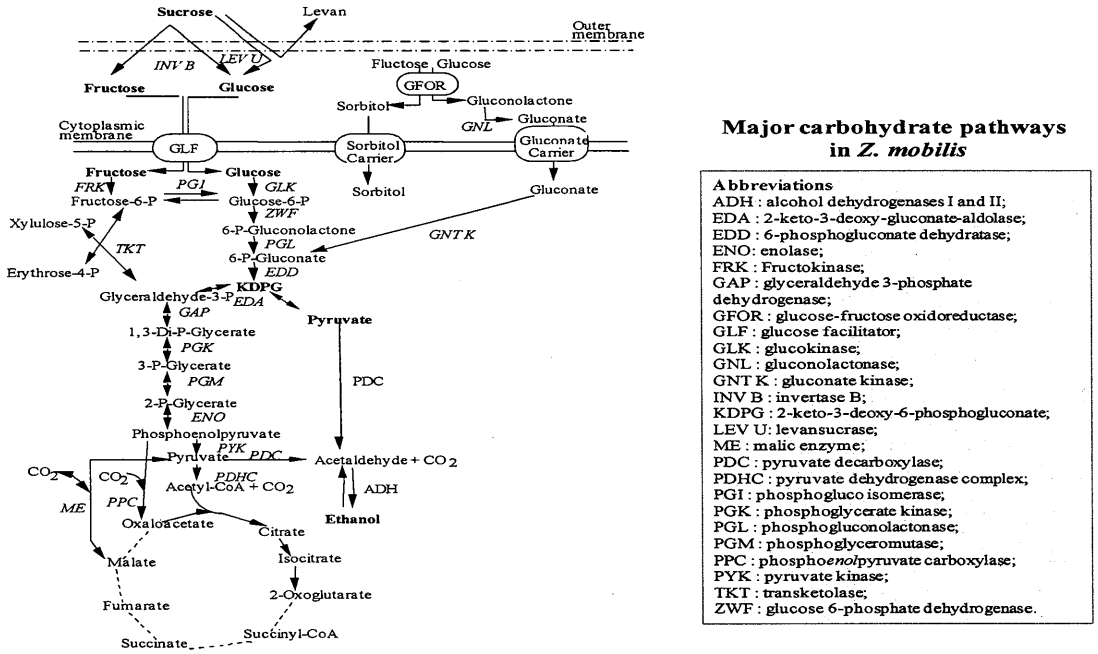
Figure 1.1 Morphology of *Zymomonas mobilis*

The genus *Zymomonas* consists of large, gram-negative rod (2-6 long and 1-4 wide), which carry out a vigorous fermentation of sugars to homo-ethanol. Although not all strains are motile, if motility occurs, it is by lophotrichous flagella. *Zymomonas* is a common organism involved in alcoholic fermentation of various plant saps, and in many tropical areas of South and Central America, Africa, and Asia, it occupies a position similar to that of *S. cerevisiae* (yeast) in North America and Europe. *Zymomonas* is involved the alcoholic fermentation of agave in Mexico, and palm sap in many tropical areas. It also carries out of ethanol fermentation from sugarcane juice and honey. Although *Zymomonas* is rarely the sole organism involved in these alcoholic fermentations, it is often the dominant organism and is probably response for the production of most of the ethanol in these beverages. *Zymomonas* is also responsible for spoilage of fruit juices such as apple cider and perry. It also may be the constituent of the bacterial flora of spoiled beer and may be responsible for the production in beer of unpleasant odor of rotten apples. *Zymomonas* resembles most closely the acetic acid bacteria, specifically *Gluconobacter* because of its polar flagellation, and it is often found in nature associated with the acetic bacteria. This is of interest because *Zymomonas* ferments glucose to ethanol, whereas the acetic acid bacteria oxidize ethanol to acetic acid. Thus, the acetic acid bacteria may depend on the activity of *Zymomonas* for the production of their growth substrate, ethanol. Like the acetic acid bacteria, *Zymomonas* is quite tolerant of low pH. Unlike yeast, which ferments glucose to ethanol via the Emden-Meyerhof (glycolytic) pathway, *Zymomonas* employs the Enter-Doudoroff pathway. This pathway is active in many pseudomonads as a means of catabolizing glucose.

*Zymomonas* is of interest to the ethanol industry because it shows higher rates of glucose uptake and ethanol production and gives a higher yield of ethanol than many

types of yeast. *Zymomonas* is also rather tolerant of high ethanol concentrations (up to 10%) but is not quite as tolerant as some of the best yeast strains, which can grow to 12-15% ethanol. However, the fact that *Zymomonas* is a Gram-negative bacterium and can thus be readily manipulated genetically makes it an attractive candidate for use by ethanol production industries.

We report the complete genome sequence of *Z. mobilis* ZM4 (ATCC31821), an ethanologenic microorganism of interest for the production of fuel ethanol. The genome consists of 2,056,416 base pairs forming a circular chromosome with 1,998 open reading frames (ORFs) and three ribosomal RNA transcription units. The genome lacks recognizable genes for 6-phosphofructokinase, an essential enzyme in the Embden-Meyerhof-Parnas pathway, and for two enzymes in the tricarboxylic acid cycle, the 2-oxoglutarate dehydrogenase complex and malate dehydrogenase, so glucose can be metabolized only by the Entner-Doudoroff pathway. Whole genome microarrays were used for genomic comparisons with the *Z. mobilis* type strain ZM1 (ATCC10988) revealing that 54 ORFs predicted to encode for transport and secretary proteins, transcriptional regulators and oxidoreductase in the ZM4 strain were absent from ZM1. Most of these ORFs were also found to be actively transcribed in association with ethanol production by ZM4 [Seo et al., 2007].




**Major carbohydrate pathways in *Z. mobilis***

**Abbreviations**
ADH : alcohol dehydrogenases I and II;
EDA : 2-keto-3-deoxy-gluconate-aldolase;
EDD : 6-phosphogluconate dehydratase;
ENO: enolase;
FRK : Fructokinase;
GAP : glyceraldehyde 3-phosphate dehydrogenase;
GFOR : glucose-fructose oxidoreductase;
GLF : glucose facilitator;
GLK : glucokinase;
GNL : gluconolactonase;
GNT K : gluconate kinase;
INV B : invertase B;
KDPG : 2-keto-3-deoxy-6-phosphogluconate;
LEV U: levansucrase;
ME : malic enzyme;
PDC : pyruvate decarboxylase;
PDHC : pyruvate dehydrogenase complex;
PGI : phosphogluco isomerase;
PGK : phosphoglycerate kinase;
PGL : phosphogluconolactonase;
PGM : phosphoglyceromutase;
PPC : phospho*enol*pyruvate carboxylase;
PYK : pyruvate kinase;
TKT : transketolase;
ZWF : glucose 6-phosphate dehydrogenase.

Figure 1.2 Major carbohydrate pathway in *Z. mobilis*

# Chapter 2

## Molecular strategy for survival at a critical high temperature in *Eschierichia coli*


### Abstract

The molecular mechanism supporting survival at a critical high temperature (CHT) in *E. coli* was investigated. Genome-wide screening with a single-gene knockout library provided a list of genes indispensable for growth at 47°C, called thermotolerant genes. Genes for which expression was affected by exposure to CHT were identified by DNA chip analysis. Unexpectedly, the former contents did not overlap with the latter except for *dnaJ* and *dnaK*, indicating that a specific set of non-heat shock genes is required for the organism to survive under such a severe condition. More than half of the mutants of the thermotolerant genes were found to be sensitive to $H_2O_2$ at 30°C, suggesting that the mechanism of thermotolerance partially overlaps with that of oxidative stress resistance. Their encoded enzymes or proteins are related to outer membrane organization, DNA double-strand break repair, tRNA modification, protein quality control, translation control or cell division. DNA chip analyses of essential genes suggest that many of the genes encoding ribosomal proteins are down-regulated at CHT. Bioinformatics analysis and comparison with the genomic information of other microbes suggest that *E. coli* possesses several systems for survival at CHT. This analysis allows us to speculate that a lipopolysaccharide biosynthesis system for outer membrane organization and a sulfur-relay system for tRNA modification have been acquired by horizontal gene transfer.


### 2.1 Introduction

Responses of *Escherichia coli* to high temperatures have been extensively investigated, though previous studies have mainly focused on the response to a temperature up-shift around 42°C, a response known as a heat shock response (HSR) to induce the expression of a set of proteins, heat-shock proteins (HSPs) [Morimoto et al.,

1996]. The fact that many HSPs are conserved among species indicates that the actions of HSRs are the fundamentally and physiologically important mechanisms in living organisms [Boorstein et al., 1994; Gupta,1995]. HSPs play crucial roles not only in the rescue or removal of proteins damaged by environmental stresses, including heat stress and salt stress, but also in the intrinsic folding of proteins under normal growth conditions [Yura et al., 2000].

It has been shown taht 384 genes are up-regulated by short-time exposure to a temperature of 43°C as a heat shock in *E. coli* [Gunasekera et al., 2008], and these genes may be directly or indirectly induced by the treatment. The directly induced genes encode HSPs, including the main cellular chaperone machineries of GroEL and DnaK, ATP-dependent proteases of Lon, HslUV, Clp and FtsH (HflB), periplasmic protease DegP, and other proteins involved in protein folding, refolding, quality control and degradation [Narberhaus et al., 1998]. HSPs are under complex regulations and are divided into several regulatory groups by their major stimulons [Erickson and Gross, 1989]. The control of their expression, however, is highly variable among organisms and even among various bacteria [Raina et al., 1995].

One of the control elements found in Gram-negative bacteria is a heat shock sigma factor that regulates transcription of the major HSPs. HSR in *E. coli* is generally mediated by alternative sigma factors, sigma 32 and sigma 24 [Yura et al., 2000; Erickson and Gross, 1989; Raina et al., 1995]. Transcription of the *rpoH* gene for sigma 32 is induced at elevated temperature via the action of sigma 24 [Erickson and Gross, 1989]. Sigma 24, which is inactive under non-stress conditions by interaction with anti-sigma factor, is activated by misfolding of outer membrane or periplasmic proteins and by stresses including heat shock [Alba et al., 2002]. Both sigma factors are further regulated at the translation level and or at the posttranslational level. The factor sigma 24 is in part regulated by a cognate small RNA, and sigma 32 synthesis is regulated by structural change of its own mRNA molecules serving as a cellular thermometer and its activity modulated by phosphorylation [Morita et al., 1999; Klein et al., 2003]. Other microorganisms, on the other hand, appear to possess diverged regulatory mechanisms [Helmann and Chamberlin 1988].

There is no information on the molecular mechanisms of response to and survival at a critical high temperature (CHT) in organisms, probably due to the limited experimental procedures. Developments of a single-gene knockout library and DNA chip analysis have encouraged us to perform a genome-wide investigation of responses in organisms under extreme conditions. Since several mesophilic bacteria including *E. coli* can grow and survive at high temperatures compared to other mesophilic bacteria,

they are assumed to have acquired the potential for thermotolerance during their evolution. In this study, we utilized new procedures for the first time to obtain information on the molecular mechanisms related to thermotolerance in *E. coli* at CHT. Screening of thermosensitive mutants at CHT and informatics analysis of the corresponding genes revealed pathways or factors indispensable for survival at CHT. For essential genes, their possible involvement in the response to CHT was examined by DNA chip analysis. Based on the results, we propose novel molecular mechanisms for survival at CHT in *E. coli.*

## 2.2 Materials and Methods

### 2.2.1 Materials

Oligonucleotide primers for polymerase chain reaction (PCR) were purchased from FASMAC Co, Ltd (Atsugi, Japan). Other chemicals were all of analytical grade.

### 2.2.2 Bacterial strains and growth conditions

Strains used in this study were derivatives of *E. coli* K-12. W3110 (IN (*rrnD-rrnE*), *rph-1*), BW25113 (*rrnB3*, *Δ(lacZ)4787*, *hsdR514*, *Δ(araBAD)567*, *Δ(rhaBAD)568, rph-1*) [Datsenko and Wanner, 2000] and mutants of BW25113 in the Keio collection as a single-gene knockout library [Baba et al., 2006] were grown on plates or in liquid of modified Luria-Bertani (LB) medium (1% Bactotryptone, 0.5% yeast extract, and 0.5% NaCl) at 37°C, 45°C or 47°C for appropriate times.

### 2.2.3 Screening of thermosensitive mutants

The Keio collection consisting of 3,908 mutant strains was used for screening. In the 1st screening, mutant strains were grown on LB plates at 30°C overnight. A colony of each strain was patched on LB plates and incubated at 47°C for 48 h to find sensitive strains. The sensitive strains were subjected to the 2nd screening of spotting tests on plates. Cells were cultured in LB medium for 18 h and then diluted with LB medium to adjust turbidity to $OD_{600}$ of 0.5, 0.05 and 0.005. The diluted samples (10 μl) were spotted on LB plates and incubated at 47°C for 48 h. The thermosensitive strains selected by the 2nd screening were subjected to the 3rd screening in liquid culture. After 8-h preculture, cells were diluted to a turbidity corresponding to $OD_{600}$ of 0.1 and

inoculated into LB medium at the final $OD_{600}$ of 0.001. Samples were then incubated at 47°C for 18 h under a shaking condition. Thermosensitivity was determined by measuring $OD_{600}$. Thermosensitive strains were defined to be <0.1 at $OD_{600}$. The experiments were performed three times, and the results were confirmed to be reproducible.

### 2.2.4 Effects of glucose and $MgCl_2$ and sensitivity to $H_2O_2$

To examine effects of supplements, glucose (0.5% (w/v)) or $MgCl_2$ (20 mM) was added to the LB liquid culture. After 8-h preculture, cells were diluted to a turbidity corresponding to $OD_{600}$ of 0.1 and inoculated into LB medium with or without the supplement at the final $OD_{600}$ of 0.001. Samples were then incubated at 47°C for 18 h under a shaking condition. After 18 h, turbidity at $OD_{600}$ was measured. To test the sensitivity to oxidative stress, $H_2O_2$ was added to the culture medium at the final concentration of 0.5 mM. After 8-h preculture, cells were diluted to a turbidity corresponding to $OD_{600}$ of 0.1 and inoculated into LB medium with or without $H_2O_2$ at the final $OD_{600}$ of 0.001. Samples were then incubated at 30°C for 8 h under a shaking condition. The experiments were performed three times, and the results were confirmed to be reproducible.

### 2.2.5 RT-PCR analysis

Cultures were grown in LB medium at 37°C until the exponential phase, and then the temperature was up-shifted to 47°C and incubation was continued for 8 min. Total RNA was immediately prepared from the heat-stressed cells by the hot phenol method [Aiba et al., 1981]. RT-PCR analysis was performed using an mRNA-selective RT-PCR kit (TAKARA BIO Inc, Otsu, Japan) to examine the expression of immediate downstream genes of disrupted genes as described previously [Tsunedomi et al., 2003]. The primer set used for each gene is shown in Table 2.1. The RT reaction was carried out at 42°C for 15 min, 85°C for 1 min, 45°C for 1 min and extension at 72°C for 2 min using the two specific primers for each gene. After the completion of 15, 20, 25 and 30 cycles, the PCR products were analyzed by 0.9% agarose gel electrophoresis and stained with ethidium bromide. The relative amounts of RT-PCR products on the gel were compared by measuring the band density after the color of the image taken had been reversed using a model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Inc, Tokyo, Japan) [Nitta et al., 2000].

**Table 2.1** RT-PCR primers used in this study.

| Name | Sequence |
|---|---|
| aceF-5' | 5'-GAGATCCTGGTCAAAGT-3' |
| aceF-3' | 5'-GCCAGCAAACGGAGCCG-3' |
| lpd-5' | 5'-AACTCAGGTCGTGGTAC-3' |
| lpd-3' | 5'-AACGGCAGTTGGATCGG-3' |
| rfaC-5' | 5'-CGATGGGCGATGTTCTC-3' |
| rfaC-3' | 5'-CATCTGTAGGCAGGTTC-3' |
| rfaF-5' | 5'-ACATGATGATGTCGCAA-3' |
| rfaF-3' | 5'-TTGTTATGGCCGCAGTT-3' |
| der-5' | 5'-GTCTAACTCGCACCCGA-3' |
| der-3' | 5'-CCGTTCTCTTCCGCTTC-3' |
| tolR-5' | 5'-GAGGTCGTCGCGATCTCA-3' |
| tolR-3' | 5'-CGGTTGGTTTAATGACGCA-3' |
| valS-5' | 5'-AAGATATCGAACAGCCG-3' |
| valS-3' | 5'-TTCACCGCATTGGACAG-3' |
| rpsL-5' | 5'-CAGTTAACCAGCTGGTA-3' |
| rpsL-3' | 5'-CTTCACGCCATACTTGG-3' |
| yheL-5' | 5'-CACATTACATCGCTCAC-3' |
| yheL-3' | 5'-TCACCAGGCCATCTGGC-3' |
| yheM-5' | 5'-CTACTGCACCTCATGGT-3' |
| yheM-3' | 5'-TCGTAGTTGGCGAGTTC-3' |
| yheN-5' | 5'-TTGCCATCGTGGTGACC-3' |
| yheN-3' | 5'-GAGCGAGGCTTCCGCCA-3' |
| dnaJ-5' | 5'-CAGCGGAAGAGCGTGAA-3' |
| dnaJ-3' | 5'-GAAGAATCCCTGGCGCA-3' |
| rpsM-5' | 5'-CCGTATAGCAGGCATTA-3' |
| rpsM-3' | 5'-TTGATCGGTTTGCGCGG-3' |
| rpsK-5' | 5'-GGCAAAGGCACCAATTCG-3' |
| rpsK-3' | 5'-TAGTGATGCGGAAACCTG-3' |
| rpsR-5' | 5'-ACGTTATTTCCGTCGTCG-3' |
| rpsR-3' | 5'-GATCAGTGTACGGCAGCA-3' |

### 2.2.6 Bioinformatics and phylogenetic analyses

Bioinformatics analysis was mainly performed according to the instructions of the KEGG site (http://www.genome.jp/kegg-bin/show_organism? menu_type=pathway maps&org=ecj). Databases of DDBJ and GenBank were also used.

### 2.2.7 DNA chip analysis

W3110 cultures were grown in LB medium at 37°C until the exponential phase, and then the temperature was up-shifted to 47°C and incubation was continued for 8 min. A control culture was incubated in parallel at 37°C for 8 min. Total RNA was immediately prepared from the heat-stressed cells by the hot phenol method [Tsunedomi et al., 2003]. Preparation of cDNA, fragmentation and the end-labeling of DNA fragments were performed according to the instruction manual from Affymetrix. The ENZO Bioarray terminal labeling kit (Enzo Life Sciences, Inc, New York, USA) was used to end-label DNA fragments. DNA hybridization, data capture and analyses were performed as described in the protocol supplied by Affymetrix and GCOS software (Affymetrix, Inc, California, USA). Two independent experiments were performed and four data sets (two data sets at 37°C: 37°C-1 and 37°C-2, two data sets at 47°C: 47°C-1 and 47°C-2) per gene were obtained. The expression ratio used here indicates the average of the ratios obtained in the two independent experiments. Spots with a significantly lower (<0.50; i.e., a negative fold difference) or higher (2>; i.e., a positive fold difference) fluorescence ratio of the heated sample to the control sample were considered to represent a real significant difference. Physiological function and functional classification of the genes were derived from the Genobase database (http://ecoli.aist-nara.ac.jp/GB6/search.jsp). Array data were submitted to ArrayExpress and the accession number will be available on the web site (http://www.ebi.ac.uk/arrayexpress/).

## 2.3 Results and Discussion

### 2.3.1 Thermosensitive mutants and thermotolerant genes

In order to identify genes required for survival at CHT in *E. coli*, we screened for thermosensitive mutants from a single-gene knockout library [Baba et al., 2006)], which had been constructed according to the one-step gene disruption method with an

*aph* cassette [Datsenko and Wanner, 2000] and for which each construct had been confirmed extensively [Yamamoto et al., 2009]. In the disrupted gene of each mutant strain, the region between the 1st codon and the last 6 codons was displaced with the *aph* cassette, so that most of the coding region of the gene was deleted. Our experiments indicated that the parental strain used for construction of the disrupted library is able to grow at temperatures up to 47°C, this temperature thus being its CHT.

After three successive screening steps of the library, including 3,908 disrupted-mutant strains, 51 strains were found to be sensitive to CHT. Their growth curves at 37°C, 45°C and 46°C were then compared to those of the parental strain (Figure S1). The growth profiles suggest that most mutants selected are significantly sensitive to 46°C and some even to 45°C. Such a disrupted gene responsible for the thermosensitive phenotype was designated as a thermotolerant gene (Table 2.2).

**Table 2.2** Thermotolerant genes identified in this study.

| Classification | Sub-classification | Gene | Function | Glc[a] | $Mg^{2+b}$ | $H_2O_2$[c] |
|---|---|---|---|---|---|---|
| Energy metabolism | Pyruvate metabolism | *aceE* | pyruvate dehydrogenase, decarboxylase component E1 | ++ | | S |
| (Group A) | Pyruvate metabolism | *aceF* | pyruvate dehydrogenase, dihydrolipoyltransacetylase component E2 | ++ | ++ | |
| | Pyruvate metabolism | *lpd* | lipoamide dehydrogenase, E3 component is part of three enzyme complexes | ++ | | |
| | Pyruvate metabolism | *lipA* | lipoate synthase | ++ | + | |
| | Propanate metabolism | *ackA* | Acetate kinase A and propionate kinase 2 | | ++ | S |
| | Pentose phosphate pathway | *rpe* | D-ribulose-5-phosphate 3-epimerase | ++ | | S |
| | Respiratory chain | *cydB* | cytochrome *d* terminal oxidase, subunit II | + | ++ | |
| | Respiratory chain | *yhcB* | cytochrome *d* terminal oxidase, subunit III | | ++ | S |
| Outer membrane | Lipopolysaccharide biosynthesis | *gmhB* | D,D-heptose 1,7-bisphosphate phosphatase | | + | |
| stabilization | Lipopolysaccharide biosynthesis | *lpcA* | D-sedoheptulose 7-phosphate isomerase | + | ++ | |
| (Group B) | Lipopolysaccharide biosynthesis | *rfaC* | ADP-heptose:LPS heptosyl transferase I | | ++ | |
| | Lipopolysaccharide biosynthesis | *rfaD* | ADP-L-glycero-D-mannoheptose-6-epimerase, NAD(P)-binding | ++ | | |
| | Lipopolysaccharide biosynthesis | *rfaE* | fused heptose 7-phosphate kinase and heptose 1 -phosphate adenyltransferase | | + | |
| | Lipopolysaccharide biosynthesis | *rfaF* | ADP-heptose:LPS heptosyltransferase II | | + | S |
| | Lipopolysaccharide biosynthesis | *rfaG* | glucosyltransferase I | + | | |
| | Peptidoglycan-associated lipoprotein | *ydcL* | predicted lipoprotein | + | + | S |
| | Peptidoglycan-associated lipoprotein | *yfgL* | protein assembly complex, lipoprotein component | ++ | S | |
| | Peptidoglycan-associated lipoprotein | *ynbE* | predicted lipoprotein | + | + | |
| | Peptidoglycan-associated lipoprotein | *nlpI* | conserved protein | + | + | |
| | Peptidoglycan-associated lipoprotein | *ycdO* | conserved protein | + | + | |
| | Outer membrane integrity | *pal* | peptidoglycan-associated outer membrane lipoprotein | ++ | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Outer membrane integrity | *tolQ* | membrane spanning protein in TolA-TolQ-TolR complex | | ++ | |
| | Outer membrane integrity | *tolR* | membrane spanning protein in TolA-TolQ-TolR complex | | ++ | S |
| | Outer membrane integrity | *yciM* | conserved hypothetical protein | + | | |
| DNA repair | DNA replication & repair, DSBR | *dnaQ* | DNA polymerase III subunit, epsilon | + | S | |
| (Group C) | DNA replication & repair, DSBR | *holC* | DNA polymerase III subunit, chi | ++ | S | |
| | DNA replication & repair, DSBR | *priA* | Primosome factor n' | ++ | + | S |
| | DNA repair, DSBR | *ruvA* | component of RuvABC resolvasome, regulatory subunit | ++ | ++ | S |
| | DNA repair, DSBR | *ruvC* | component of RuvABC resolvasome, endonuclease | + | + | S |
| tRNA modification | tRNA modification | *iscS* | sulfer relay system, cysteine desulfurase | | | S |
| (Group D) | tRNA modification | *yheL* | sulfer relay system, predicted intracellular sulfur oxidation protein | | S | |
| | tRNA modification | *yheM* | sulfer relay system, predicted intracellular sulfur oxidation protein | | S | |
| | tRNA modification | *yheN* | sulfer relay system, predicted intracellular sulfur oxidation protein | | S | |
| | tRNA modification | *yhhP* | conserved protein required for cell growth | | | S |
| | tRNA modifoication | *miaA* | delta(2)-isopentenylpyrophosphate tRNA-adenosine transferase | + | S | |
| | tRNA modification | *trmU* | tRNA (5-methylaminomethyl-2-thiouridylate)- methyltransferase | | S | |
| | tRNA modification | *truA* | pseudouridylate synthase I | + | | |
| Chaperone/protease | Chaperon system | *dnaJ* | chaperone Hsp40, co-chaperone with DnaK | | ++ | S |
| (Group E) | Chaperon system | *dnaK* | chaperone Hsp70, co-chaperone with DnaJ | | | S |
| | Chaperon system | *degP* | Chaperone/serine endoprotease | | | |
| | Chaperon regulator | *rseA* | Anti-sigma factor | ++ | S | |
| Translation control | Translation control | *rpmJ* | 50S ribosomal subunit L36, related to secY expression | | | S |
| (Group F) | Translation control | *rpsF* | 30S ribosomal subunit S6, supecifically modified with glutamic acid or phosphate | | S | |
| | Translation control | *dksA* | DNA-binding transcriptional regulator or rRNA transcription DnaK suppressor | + | + | S |

| | | | | | | |
|---|---|---|---|---|---|---|
| | Translation control | *smpB* | Component of trans-translation process | + | + | S |
| Cell division | Related to cell division | *xerC* | Site-specific tyrosine recombinse involved in chromosome dimer resolution | ++ | S | |
| (Group G) | Related to cell division | *dedD* | Membrane-anchored periplasmic protein involved in separation | ++ | | |
| | Related to cell division | *envC* | Regulator of cell wall hydrolases responsible for cell separation | + | | |
| Others | Membrane transport | *zntA* | Zinc/cadmium/mercury/lead-exporting ATPase | + | + | |
| | Membrane transport | *ybgH* | Predicted proton-dependent oligopeptide Transporter, POT family | + | ++ | |
| | Membrane transport | *ybhH* | Conserved hypothetical protein | ++ | ++ | S |

[a]According to the data in Figure S4, ratios of growth in the presence of glucose to that in the absence of glucose at 46°C were estimated. "++" and "+" represent more than 2.0 and 1.5-2.0, respectively.

[b]According to the data in Figure S4, ratios of growth in the presence of $MgCl_2$ to that in the absence of $MgCl_2$ at 46°C were estimated. "++" and "+" represent more than 2.0 and 1.5-2.0, respectively.

[c]According to the data in Figure S4, ratios of growth in the presence of $H_2O_2$ to that in the absence of $H_2O_2$ at 30°C were estimated. "S" represents less than 0.5.

The gene organization generated by construction of the disrupted mutants might give rise to a polar effect of the inserted *aph* gene on transcription of downstream genes that are intrinsically transcribed by read-through from the promoter or the region upstream of the disrupted gene. Such an organization was found in 42 of the 51 mutants. Sensitivity was not due to a polar effect in 29 of those 42 mutants because disruption of genes just downstream from the disrupted gene by the same method caused no thermosensitive phenotype. The remaining 13 mutants have either an essential gene or a thermotolerant gene as an immediate downstream gene (Figure S2). Their possible polar effects were thus tested by RT-PCR with total RNA prepared from cells exposed to a temperature of 37°C or 47°C (Figure S3). The results suggest that the transcription level of the immediate downstream gene in the mutant was almost the same as that in the parent in all cases except for the cases of mutants of *aceF*, *tolQ*, *dnaK* and *rpsF*. Most of these downstream genes would thus have their own promoters or the transcription level by read-through would be nearly the same as that of the *aph* promoter. However, the transcription levels of *lpd*, *tolR* and *dnaJ* located downstream of *aceF*, *tolQ* and *dnaK*, respectively, were increased and the level of *rpsR* located downstream of *rpsF* was decreased compared to those of the parental strain at both temperatures. Although the expressional alteration of the 4 genes was nearly the same at both temperatures, growth of the corresponding mutant strains at 37°C was not significantly changed from that of the parental strain. Taken together, the results suggest that the thermotolerant phenotype in the 51 mutants is due to disruption of the targeted gene and not due to a polar effect on its downstream genes. Out of the 51 thermotolerant genes, 8 genes, *cydB, degP, dnaJ, dnaK, dnaQ, nlpI, rfaD* and *rfaC*, had been reported as genes supporting growth at a high temperature. [Wall et al., 1992; Raina and Georgopoulos 1991; Klein et al., 2009; Adams and West 1996; Tomoyasu et al., 1998; Lipinska et al., 1990; Ohara et al., 1999], and thus we newly identified 43 thermotolerant genes in this organism.

### 2.3.2 Effects of supplements and oxidative stress on growth of thermosensitive mutant strains

Since LB was utilized as a medium for the screening of thermosensitive mutants, limitation of carbon source might cause sensitiveness to CHT. We thus examined the effect of glucose as a supplement for growth of the thermosensitive mutant strains (Table 2.2, Figure S4). We also tested the effect of $MgCl_2$ because $Mg^{2+}$ somehow protects against cell damage under stress conditions [Kabir et al., 2005; Noor et al.,

2009]. The growth of 20 and 37 mutants was improved at CHT by the addition of 0.5% glucose and 20 mM $MgCl_2$, respectively. The growth of sixteen mutant strains was improved by supplementation of not only glucose but also $MgCl_2$.

Next, the effect of exogenous oxidative stress on the thermosensitive mutant strains was tested since a higher temperature causes more oxidative stress (Noor *et al*, 2009; unpublished data). We exposed thermosensitive mutant strains to 0.5 mM $H_2O_2$ in LB liquid medium at 30°C. Twenty-nine mutants were found to be sensitive to $H_2O_2$ (Table 2.2, Figure S4), corresponding to approximately 60% of the thermosensitive mutants. Moreover, out of the 10 thermosensitive mutants for which glucose and $MgCl_2$ supplementation had no effect, 9 mutants showed sensitivity to $H_2O_2$. These results suggest that the mechanism of thermotolerance at CHT partially overlaps with that of oxidative stress resistance.

### 2.3.3 Bioinformatics analysis and classification of thermotolerant genes

To understand the molecular mechanism of *E. coli* survival at CHT, bioinformatics analysis with various public databases including the KEGG PATHWAY database was performed. Out of the 51 thermotolerant genes, 29 genes were successfully mapped on *E. coli* pathways in the KEGG PATHWAY database. Interestingly, many genes were found to be involved in the same metabolic pathway, suggesting that the organism possesses indispensable pathways at CHT. The remaining 19 genes except for 3 unknown genes were extensively analyzed by using the DDBJ or GenBank database. On the basis of results of these analyses and the effects of the supplements, the 51 thermotolerant genes were classified into 7 groups (Table 2.2).

Group A consists of genes concerned with energy metabolism for production of ATP. The gene products of *aceE*, *aceF*, *lpd* and *ackA* are mapped in the pyruvate metabolism pathway from pyruvate to acetyl CoA [Haydon et al., 1993; Lyngstadaas et al., 1995; Cassey et al., 1988; Barak et al., 1998] and that of *rpe* is located in the pentose phosphate pathway. *cydB* and *yhcB* encode subunits of cytochrome *d* terminal oxidase, which generates the membrane potential responsible for ATP synthesis [Wall et al., 1992; Mogi et al., 2006]. *lipA*, which encodes LipA to produce lipoate required for pyruvate dehydrogenase reaction, also contributes to pyruvate metabolism [Reed and Cronan, 1993]. Based on the results showing that disrupted mutations of these genes caused a thermosensitive phenotype, we assumed that the cells require more ATP at a higher temperature. This assumption was supported by the finding that the

phenotype of most mutants in this group was partially suppressed by the addition of glucose (Table 2.2, Figure S4).

Group B consists of genes related to biosynthesis of the cell wall or organization of the outer membrane. The products of *gmhB, lpcA(gmhA), rfaC* (*waaC*), *rfaD* (*waaD/htrM*), *rfaE* (*gmhC*), *rfaF* (*waaF*) and *rfaG* (*waaG*) were mapped into the lipopolysaccharide (LPS) biosynthesis pathway [Raina and Georgopoulos, 1991; Klein et al., 2009; Kneidinger et al., 2002; Roncero and Casadaban, 1992]. The products of these genes are involved in synthesis of the heptose unit of ADP-L-glycero-D-manno-heptose from sedoheptulose-7phosphate or encode early heptosyl transferases for KDO-lipid A (*rfaC* and *rfaF*) and to further extend the inner core of LPS with glcosyltransferase (*rfaG*). *ydcL*, *yfgL* (*bamB*), *ynbE*, *nlpI* and *ycdO* encode peptidoglycan-associated outer membrane lipoproteins, and the products of *pal*, *tolQ* and *tolR* are components for a complex structure forming a biopolymer transporter [Gerding et al., 2007; Kampfenkel and Braun, 1993]. *yciM* encodes a protein possibly required for integrity of the outer membrane [Niba ETet al., 2007]. The thermosensitive phenotype caused by disrupted mutants of all of these genes was significantly suppressed by the addition of $Mg^{2+}$ (Table 2.2, Figure S4). Since $Mg^{2+}$ is known to stabilize the outer membrane structure by binding extracellularly [Nikaido, 2003], it is assumed that YdcL, YfgL, YnbE, NlpI, YcdO, Pal, TolQ, TolR and YciM act as components or scaffold proteins of the membrane to maintain outer membrane integrity, especially at a high temperature. Similarly, our data suggest that $Mg^{2+}$ is able to stabilize the outer membrane structure when the LPS biosynthesis pathway becomes defective.

Group C consists of *dnaQ*, *holC*, *priA, ruvA* and *ruvC* for DNA double-strand break repair (DSBR) [Motamedi et al., 1999]. DnaQ and HolC are epsilon and chi subunits, respectively, of DNA polymerase III [Fijalkowska et al., 1997;39 Xiao et al., 1993], which is required for homologous recombination in DSBR [Adams and West, 1996]. RuvA and RuvC act as DNA helicase and endonuclease, respectively [Adams and West, 1996; 40 Bennett and West, 1996], before the replication restart in the DSBR process, and PriA functions as DNA helicase after the replication restart [Al-Deib et al., 1996]. The requirement of DSBR for survival at CHT suggests that DNA molecules are subjected more to double-strand breaks at a higher temperature. Interestingly, mutants of all members in this group exhibited sensitivity to oxidative stress at 30°C. Therefore, it is thought that there is a strong connection between oxidative stress and DNA double-strand breaks.

Group D includes genes for tRNA modification. Products of *iscS*, *yheL* (*tusB*), *yheM* (*tusC*), *yheN* (*tusD*) and *yhhP* (*tusA*) have been demonstrated to compose the sulfur-relay system [Rojas and Vásquez, 2005; Ikeuchi et al., 2006; Dahl et al., 2008]. IscS is a widely distributed cysteine desulfurase that catalyzes desulfuration of L-cysteine by transfer of the sulfur to its active-site cysteine to form a persulfide group (-SSH), being responsible together with YheL, YheM, YheN and YhhP for biosynthesis of the 2-thio modification of 5-methylaminomethyl-2-thiouridine ($mnm^5s^2U$) [Ikeuchi et al., 2006] and five different thio modifications in bacterial tRNAs [Lauhon, 2002]. IscS also works as a general sulfur donor in various metabolic pathways [Mihara and Esaki, 2002] including biosynthesis of iron-sulfur (Fe-S) cluster [Frazzon and Dean, 2003], thiamine [Taylor et al., 1998], nicotinic acid and branched-chain amino acids [Lauhon and Kambampati, 2000]. Additionally, *miaA*, *trmU* and *truA* in this group are involved in tRNA modification. The mutations of genes related to sulfer modification cause the phenotype of sensitivity to anti-oxidation stress [Dahl et al., 2008]. Consistently, our study provided evidence that mutants of this group exhibited hypersensitivity to oxidative stress. YheL, YheM, YheN and YhhP, which mainly function in t-RNA modification [Lauhon, 2002], are conserved in thermotolerant bacteria in mesophiles (see Table 2.3), whereas *iscS*, a general sulfur donor, is widely conserved in mesophiles. These findings suggest that tRNA modifications presented here are indispensable for growth at CHT.

**Table 2.3** Distribution of thermotolerant genes in group D in various bacteria.

| Bacteria[a] | *iscS* | *yheL* | *yheM* | *yheN* | *yhhP* | *miaA* | *truU* | *truA* |
|---|---|---|---|---|---|---|---|---|
| *Escherichia coli* | o | o | o | o | o | o | o | o |
| *Salmonella enterica* | o | o | o | o | o | o | o | o |
| *Yersinia pestis* | o | o | o | o | o | o | o | o |
| *Shigella flexneri* | o | o | o | o | o | o | o | o |
| *Klebsiella pneumoniae* | o | o | o | o | o | o | o | o |
| *Xanthomonas campestris* | x | x | x | x | x | o | o | o |
| *Xanthomonas axonopodis* | x | x | x | x | x | o | o | o |
| *Vibrio cholerae* | o | o | o | o | o | o | o | o |
| *Pseudomonas aeruginosa* | o | o | o | o | o | o | o | o |
| *Pseudomonas putida* | o | o | o | o | o | o | o | x |
| *Pseudomonas syringae* | o | o | o | o | o | o | o | o |

| Bacteria[a] | *iscS* | *yheL* | *yheM* | *yheN* | *yhhP* | *miaA* | *trmU* | *truA* |
|---|---|---|---|---|---|---|---|---|
| *Azotobacter vinelandii* | o | o | o | o | o | o | o | o |
| *Acinetobacter sp* | o | x | x | o | o | o | o | o |
| *Neisseria meningitides* | o | x | x | x | x | o | o | o |
| *Nitosomonas europaea* | o | x | x | x | x | o | o | o |
| *Helicobacter pylor* | o | x | x | x | x | o | o | o |
| *Campylobacter jejuni* | o | x | x | x | x | o | o | o |
| *Geobacter sulfurreducens* | o | x | x | x | x | o | o | o |
| *Rickettsia prowazekii* | o | x | x | x | x | o | o | o |
| *Agrobacterium tumefaciens* | o | x | x | x | x | o | o | o |
| *Rhizobium etli* | o | x | x | x | x | o | o | o |
| *Brucella melitensis* | o | x | x | x | x | o | o | o |
| *Rhodopseudomonas palustris* | o | x | x | x | x | o | o | o |
| *Methylobacterium extorquens* | o | x | x | x | x | o | o | o |
| *Caulobacter crescentus* | o | x | x | x | x | o | o | o |
| *Rhodobacter sphaeroides* | o | x | x | x | o | o | o | o |
| *Zymomonas mobilis* | o | x | x | x | x | o | o | o |
| *Gluconacetobacter diazotrophicus* | o | x | x | x | x | o | o | o |
| *Acetobacter pasteurianus* | o | x | x | x | x | o | o | o |
| *Bacillus subtilis* | o | x | x | x | x | o | o | o |
| *Bacillus cereus* | o | x | x | x | x | o | o | o |
| *Bacillus licheniformis* | o | x | x | x | x | o | o | o |
| *Staphylococcus aureus* | o | x | x | x | x | o | o | o |
| *Lactococcus lactis* | o | x | x | x | x | o | o | o |
| *Streptococcus pyogenes* | o | x | x | x | x | o | o | o |
| *Lactobacillus plantarum* | o | x | x | x | x | o | o | o |
| *Clostridium acetobutylicum* | o | x | x | x | x | o | o | o |
| *Mycoplasma genitalium* | x | x | x | x | x | x | x | o |
| *Mycobacterium tuberculosis* | o | x | x | x | x | o | o | o |
| *Corynebacterium glutamicum* | o | x | x | x | x | o | o | o |
| *Corynebacterium efficiens* | o | x | x | x | x | o | o | o |
| *Streptomyces coelicolor* | o | x | x | x | x | o | o | o |
| *Chlamydia trachomatis* | o | x | x | x | x | o | o | o |
| *Chlamydophila pneumoniae* | o | x | x | x | x | o | o | o |

| Bacteria[a] | *iscS* | *yheL* | *yheM* | *yheN* | *yhhP* | *miaA* | *trmU* | *truA* |
|---|---|---|---|---|---|---|---|---|
| *Borrelia burgdorferi* | o | x | x | x | x | x | x | o |
| *Flavobacterium johnsoniae* | o | x | x | x | x | o | o | o |
| *Flavobacterium psychrophilum* | o | x | x | x | x | o | o | o |
| *Synechocystis sp* | o | x | x | x | x | o | o | o |
| *Chlorobaculum tepidum* | o | x | o | o | o | o | o | o |
| *Chlorobium chlorochromatii* | o | o | o | o | o | o | o | o |
| *Deinococcus radiodurans* | o | x | x | x | x | o | o | o |
| *Thermotoga maritime* | o | o | x | x | x | o | o | o |
| *Archaeoglobus fulgidus* | o | x | x | x | x | x | x | o |
| *Pyrococcus horikoshii* | x | x | x | x | x | x | x | o |
| *Methylococcus capsulatus* | o | o | x | o | x | o | o | o |
| *Methanococcus jannaschii* | x | x | x | x | x | x | x | o |
| *Methanobacterium thermoautotrophicum* | x | o | o | x | x | x | x | o |
| *Halobacterium sp* | x | x | x | x | x | x | x | o |
| *Thermoanaerobacter tengcongensis* | o | o | o | o | x | o | o | o |
| *Thermodesulfovibrio yellowstonii* | o | x | x | x | x | o | o | o |
| *Thermanaerovibrio acidaminovorans* | o | x | x | x | x | o | o | o |

[a]Bacteria shown here are representatives of species of which genomic sequences are available in databases. "o" and "x" represent the presence and absence of themotorelant genes in group D, respectively.

Group E genes encode chaperones and a protease and thus contribute to the cellular process of regulating heat shock response: *dnaK* and *dnaJ* encode a chaperone and co-chaperone, respectively, for maturation of protein folding or refolding of unfolded proteins [Tomoyasu et al., 1998], and *degP* encodes a chaperone/serine protease located in the periplasm [Lipinska et al.,1990]. The indispensability of these genes at CHT suggests that DnaK/DnaJ play a crucial role in dealing with unfolded proteins caused by CHT and that DegP plays an important role in the removal of damaged proteins that have accumulated at such a temperature. *rseA* in this group encodes an anti-sigma factor to keep sigma 24 inactive under non-stress conditions. The thermosensitivity caused by *rseA* disrupted mutation suggests that fine tuning of the intracellular level of active sigma 24 that regulates expression of chaperone or protease

genes is somehow crucial for adaptation to the CHT condition. Alternatively, the defective mutant of *rseA* increased sigma 24 activity, which in turn decreased the production of outer membrane proteins via MicA or RybB as a sigma 24 regulon gene [Valentin-Hansen et al., 2007], resulting in membrane unstability and thermosensitiveness at CHT.

Genes in group F belong to the translation control apparatus. S6 encoded by *rpsF* interacts with the central domain of 16S rRNA and has been demonstrated to play a regulatory rather than a structural role in the ribosome [Britton and Lupski et al., 1997]. L36 encoded by *rpmJ* is a component of the 50S subunit of the ribosome, and its disruption decreases the expression of *secY* [Ikegami et al., 2005], which encodes a protein-conducting channel in the cytoplasmic membrane. DksA encoded by *dksA* functions as a negative regulator for rRNA genes [Perron et al., 2005]. Overexpression of DksA has been shown to be a suppressor for a *dnaK* deletion mutation [Kang and Craig,1990] and ensures replication completion by removing transcription roadblocks [Tehranchi, 2010]. SmpB encoded by *smpB* is a component of the trans-translation process and performs rescue of stalled ribosomes with its binding partner, transfer-messenger RNA [Watts et al., 2009]. These lines of evidence suggest that several constituents in translation pathways are crucial for survival at CHT.

Finally, genes in group G are related to cell division. A *xerC*-encoded protein is a site-specific recombinase [Bloor and Cranenburgh, 2006] and is essential for conversion of chromosome dimers to monomers during cell division. *envC* encodes a component of the cell division machinery that is a direct regulator of the cell wall hydrolase responsible for cell separation that is required for cell division [Uehara et al., 2010]. DedD encoded by *dedD* is a membrane-anchored periplasmic protein involved in septation [Gerding et al., 2009] and has been shown to participate in cytokinesis [Arends et al., 2010].

The functions of the remaining genes, *ybgH, yciM* and *yhhH,* are unknown. Notably, the thermosensitiveness of their mutations was partially suppressed by the addition of $Mg^{2+}$. It is thus likely that their gene products are related to cellular activities similar to those in group B, C or G.

### 2.3.4 Possible acquisition of some thermotolerant genes by horizontal gene transfer

Two groups for outer membrane integrity and tRNA modification are almost completely conserved in limited bacterial species with optimal growth at a relatively high temperature (Table 2.4). Of these group members, genes for the LPS biosynthesis

pathway, some lipoproteins and the sulfur-relay system are distributed in very limited bacterial species including *Enterobacteriaceae* (Tables 2.5 and 2.3). The sulfur-relay system classified in tRNA modification has been demonstrated to modify a few nucleotides of tRNA molecules, contributing to stabilization of their structure, and to be required for survival at an extremely high temperature in *Thermous thermophilus* [Shigi et al., 2008] and it is also conserved in *Thermoanaerobacter tengcongensis* (Table 2.3). The mature LPS biosynthesis pathway for assembly of the outer membrane consists of many enzyme reactions, which was found to be dispensable at a lower temperature. Interestingly, this pathway is mostly conserved in *Thermodesulfovibrio yellowstonii* and *Thermanaerovibrio acidaminovorans* (Table 2.5). Enzymes in the LPS biosynthesis and sulfur-relay system in *E. coli* share about 40% sequence identity and about 50% sequence similarity to the corresponding enzymes in thermophilic bacteria. *E. coli* and its closely related bacteria would thus have acquired these genes of the two groups presumably by horizontal gene transfer during their evolution. Since the other five groups are widely conserved not only in thermotolerant mesophilic bacteria but also in other mesophilic bacteria, they would be intrinsically present in *E. coli.* This is consistent with the conserved nature of essentiality of the lipid A part of LPS and essentiality of synthesis of lipid $IV_A$ but dispensability of enzymes involved in extension of $Kdo_2$-lipid A by various glycosyltransferases. This draws support from Re (*rfaC*) mutants with only tetraacylated lipid A exhibiting a very narrow growth range with ability to grow only under slow growth conditions on minimal medium around 23°C [Klein et al., 2009], suggesting overall importance of outer membrane integrity at CHT.

**Table 2.4**. Distribution of thermotolerant genes in various bacteria.

| Bacteria[a] Temperature | Optimum Metabolism (°C) | Energy membrane stabilization (Group A) | Outer repair (Group B) | DNA modification (Group C) | tRNA protease (Group D) | Chaperone/ control (Group E) | Translation division (Group F) | Cell (Group G) |
|---|---|---|---|---|---|---|---|---|
| | | 8 genes | 16 genes | 5 genes | 8 genes | 4 genes | 4 genes | 3 genes |
| *Escherichia coli* | 25-39 | 8 | 16 | 5 | 8 | 4 | 4 | 3 |
| *Salmonella enterica* | 30-39 | 8 | 15 | 5 | 8 | 4 | 4 | 3 |
| *Yersinia pestis* | 28-35 | 8 | 14 | 5 | 8 | 4 | 4 | 3 |
| *Shigella flexneri* | 30-35 | 8 | 16 | 5 | 8 | 4 | 4 | 3 |
| *Klebsiella pneumoniae* | | 8 | 16 | 5 | 8 | 4 | 4 | 3 |
| *Xanthomonas campestris* | | 6 | 6 | 5 | 3 | 2 | 4 | 2 |
| *Xanthomonas axonopodis* | | 6 | 6 | 5 | 3 | 2 | 4 | 3 |
| *Vibrio cholerae* | 30-40 | 8 | 10 | 5 | 8 | 4 | 4 | 3 |
| *Pseudomonas aeruginosa* | | 8 | 13 | 5 | 8 | 4 | 4 | 3 |
| *Pseudomonas putida* | 25-30 | 7 | 12 | 5 | 7 | 4 | 4 | 3 |
| *Pseudomonas syringae* | 25-30 | 8 | 12 | 5 | 8 | 4 | 4 | 3 |
| *Azotobacter vinelandii* | | 8 | 13 | 5 | 8 | 4 | 4 | 3 |
| *Acinetobacter sp* | 33-35 | 8 | 6 | 5 | 6 | 2 | 4 | 2 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Neisseria meningitidis* | 35-37 | 6 | 8 | 5 | 4 | 2 | 4 | 2 |
| *Nitosomonas europaea* | | 6 | 7 | 5 | 4 | 3 | 3 | 3 |
| *Helicobacter pylori* | 30-37 | 2 | 8 | 4 | 4 | 2 | 3 | 1 |
| *Campylobacter jejuni* | 35-37 | 5 | 9 | 4 | 4 | 2 | 4 | 2 |
| *Geobacter sulfurreducens* | | 6 | 9 | 4 | 4 | 2 | 2 | 2 |
| *Rickettsia prowazekii* | | 5 | 4 | 5 | 4 | 2 | 4 | 2 |
| *Agrobacterium tumefaciens* | 25-28 | 5 | 4 | 5 | 4 | 2 | 4 | 2 |
| *Rhizobium etli* | 25-30 | 5 | 4 | 5 | 4 | 2 | 4 | 2 |
| *Brucella melitensis* | | 5 | 4 | 5 | 4 | 2 | 4 | 2 |
| *Rhodopseudomonas palustris* | 30-37 | 6 | 10 | 5 | 4 | 2 | 4 | 2 |
| *Methylobacterium extorquens* | 25-30 | 6 | 4 | 5 | 4 | 2 | 4 | 2 |
| *Caulobacter crescentus* | 25-30 | 6 | 6 | 5 | 4 | 2 | 4 | 2 |
| *Rhodobacter sphaeroides* | 30-34 | 7 | 4 | 5 | 5 | 2 | 4 | 2 |
| *Zymomonas mobilis* | 25-30 | 5 | 4 | 5 | 4 | 2 | 4 | 2 |
| *Gluconacetobacter diazotrophicus* | | 6 | 6 | 5 | 4 | 2 | 4 | 2 |
| *Acetobacter pasteurianus* | | 6 | 6 | 5 | 4 | 2 | 3 | 2 |
| *Bacillus subtilis* | 28-30 | 6 | 4 | 2 | 4 | 2 | 3 | 2 |
| *Bacillus cereus* | ~37 | 6 | 2 | 3 | 4 | 2 | 3 | 2 |
| *Bacillus licheniformis* | | 6 | 2 | 2 | 4 | 2 | 3 | 2 |
| *Staphylococcus aureus* | 30-37 | 6 | 3 | 3 | 4 | 2 | 3 | 2 |
| *Lactococcus lactis* | | 5 | 2 | 3 | 4 | 2 | 3 | 1 |
| *Streptococcus pyogenes* | | 4 | 3 | 3 | 4 | 2 | 3 | 1 |
| *Lactobacillus plantarum* | 30-40 | 5 | 3 | 3 | 4 | 2 | 3 | 2 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Clostridium acetobutylicum* | | 2 | 4 | 3 | 4 | 2 | 3 | 1 |
| *Mycoplasma genitalium* | | 4 | 0 | 1 | 1 | 2 | 3 | 1 |
| *Mycobacterium tuberculosis* | | 7 | 3 | 4 | 4 | 2 | 3 | 2 |
| *Corynebacterium glutamicum* | | 6 | 3 | 4 | 4 | 2 | 2 | 2 |
| *Corynebacterium efficiens* | | 6 | 3 | 4 | 4 | 2 | 3 | 2 |
| *Streptomyces coelicolor* | 25-35 | 7 | 4 | 4 | 4 | 2 | 4 | 1 |
| *Chlamydia trachomatis* | 37 | 5 | 4 | 4 | 4 | 2 | 4 | 2 |
| *Chlamydophila pneumoniae* | 37 | 5 | 4 | 4 | 4 | 2 | 4 | 2 |
| *Borrelia burgdorferi* | | 1 | 2 | 2 | 2 | 2 | 3 | 1 |
| *Flavobacterium johnsoniae* | | 6 | 4 | 4 | 4 | 2 | 4 | 2 |
| *Flavobacterium psychrophilum* | | 4 | 3 | 4 | 4 | 2 | 3 | 2 |
| *Synechocystis sp* | | 6 | 3 | 3 | 4 | 2 | 3 | 2 |
| *Chlorobaculum tepidum* | | 5 | 7 | 4 | 7 | 2 | 3 | 1 |
| *Chlorobium chlorochromatii* | 25-30 | 4 | 8 | 3 | 8 | 2 | 3 | 1 |
| *Deinococcus radiodurans* | | 6 | 2 | 4 | 4 | 2 | 3 | 1 |
| *Thermotoga maritime* | | 3 | 2 | 4 | 5 | 2 | 3 | 1 |
| *Archaeoglobus fulgidus* | | 1 | 2 | 1 | 2 | 0 | 0 | 1 |
| *Pyrococcus horikoshii* | | 0 | 2 | 0 | 1 | 0 | 0 | 1 |
| *Methylococcus capsulatus* | ~45 | 7 | 8 | 5 | 6 | 3 | 4 | 3 |
| *Methanococcus jannaschii* | 35-40 | 2 | 3 | 0 | 1 | 0 | 0 | 1 |
| *Methanobacterium thermoautotrophicum* | 37-45 | 1 | 2 | 0 | 3 | 1 | 0 | 1 |
| *Halobacterium sp* | 35-50 | 4 | 2 | 0 | 1 | 2 | 0 | 1 |
| *Thermoanaerobacter tengcongensis* | 55-75 | 5 | 3 | 3 | 7 | 2 | 3 | 1 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Thermodesulfovibrio yellowstonii* | 60–65 | 4 | 11 | 4 | 4 | 2 | 3 | 2 |
| *Thermanaerovibrio acidaminovorans* | 60–65 | 1 | 9 | 4 | 4 | 2 | 3 | 2 |

[a]Bacteria shown here are representatives of species of which genomic sequences are available in databases.

**Table 2.5** Distribution of thermotolerant genes in group B in various bacteria.

| Bacteria[a] | *gmhB* | *lpcA* | *rfaC* | *rfaD* | *rfaE* | *rfaF* | *rfaG* | *ydcL* | *yfgL* | *ynbE* | *nlpI* | *ycdO* | *pal* | *tolQ* | *tolR* | *yciM* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Escherichia coli* | o | o | o | o | o | o | o | o | o | o | o | o | o | o | o | o |
| *Salmonella enterica* | o | o | o | o | o | o | o | o | o | o | o | x | o | o | o | o |
| *Yersinia pestis* | o | o | o | o | o | o | x | x | o | o | o | o | o | o | o | o |
| *Shigella flexneri* | o | o | o | o | o | o | o | o | o | o | o | o | o | o | o | o |
| *Klebsiella pneumoniae* | o | o | o | o | o | o | o | o | o | o | o | o | o | o | o | o |
| *Xanthomonas campestris* | x | x | x | x | x | x | x | o | o | x | x | x | o | o | o | o |
| *Xanthomonas axonopodis* | x | x | x | x | x | x | x | o | o | x | x | x | o | o | o | o |
| *Vibrio cholerae* | o | o | x | o | x | o | x | x | o | x | o | x | o | o | o | o |
| *Pseudomonas aeruginosa* | o | o | o | o | o | o | o | x | o | o | x | x | o | o | o | o |
| *Pseudomonas putida* | o | o | o | x | o | o | o | o | o | o | x | x | x | o | o | o |
| *Pseudomonas syringae* | o | o | o | x | o | o | o | x | o | x | x | o | o | o | o | o |
| *Azotobacter vinelandii* | o | o | o | x | o | o | o | o | o | o | x | x | o | o | o | o |
| *Acinetobacter sp* | x | x | x | x | x | x | x | o | o | x | x | x | o | o | o | o |
| *Neisseria meningitides* | o | o | o | o | x | o | x | x | o | x | x | o | x | x | x | o |
| *Nitosomonas europaea* | o | x | x | x | x | x | x | o | o | x | x | x | o | o | o | o |
| *Helicobacter pylori* | o | o | o | o | o | o | x | x | x | x | x | x | o | x | x | o |
| *Campylobacter jejuni* | o | o | o | o | o | o | x | x | o | x | x | x | o | x | x | o |

| Bacteria[a] | *gmhB* | *lpcA* | *rfaC* | *rfaD* | *rfaE* | *rfaF* | *rfaG* | *ydcL* | *yfgL* | *ynbE* | *nlpI* | *ycdO* | *pal* | *tolQ* | *tolR* | *yciM* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Geobacter sulfurreducens* | o | o | o | x | o | o | x | x | o | x | x | x | o | o | x | o |
| *Rickettsia prowazekii* | x | x | x | x | x | x | x | x | o | x | x | x | o | o | x | o |
| *Agrobacterium tumefaciens* | x | x | x | x | x | x | x | x | o | x | x | x | o | o | x | o |
| *Rhizobium etli* | x | x | x | x | x | x | x | x | o | x | x | x | o | o | x | o |
| *Brucella melitensis* | x | x | x | x | x | x | x | x | o | x | x | x | o | o | x | o |
| *Rhodopseudomonas palustris* | o | o | o | o | o | o | o | x | o | x | x | x | x | o | x | o |
| *Methylobacterium extorquens* | x | x | x | x | x | x | x | x | o | x | x | x | o | o | x | o |
| *Caulobacter crescentus* | x | x | x | o | o | x | x | x | o | x | x | x | o | o | x | o |
| *Rhodobacter sphaeroides* | x | x | x | x | x | x | x | x | o | x | x | x | o | o | x | o |
| *Zymomonas mobilis* | x | x | x | x | x | x | x | x | o | o | x | x | o | o | x | o |
| *Gluconacetobacter diazotrophicus* | x | x | x | o | o | x | x | x | o | x | x | x | o | o | x | o |
| *Acetobacter pasteurianus* | x | x | x | o | o | x | x | x | o | x | x | x | o | o | x | o |
| *Bacillus subtilis* | x | x | x | x | x | x | x | o | o | x | x | o | x | x | x | o |
| *Bacillus cereus* | x | x | x | x | x | x | x | x | o | x | x | x | x | x | x | o |
| *Bacillus licheniformis* | x | x | x | x | x | x | x | x | o | x | x | x | x | x | x | o |
| *Staphylococcus aureus* | x | x | x | x | x | x | x | o | o | x | x | x | x | x | x | o |
| *Lactococcus lactis* | x | x | x | x | x | x | x | x | o | x | x | x | x | x | x | o |
| *Streptococcus pyogenes* | x | x | x | x | x | x | x | o | o | x | x | x | x | x | x | o |

| Bacteria[a] | *gmhB* | *lpcA* | *rfaC* | *rfaD* | *rfaE* | *rfaF* | *rfaG* | *ydcL* | *yfgL* | *ynbE* | *nlpI* | *ycdO* | *pal* | *tolQ* | *tolR* | *yciM* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Lactobacillus plantarum* | x | x | x | x | x | x | x | o | o | x | x | x | x | x | x | o |
| *Clostridium acetobutylicum* | o | o | x | x | x | x | x | x | o | x | x | x | x | x | x | o |
| *Mycoplasma genitalium* | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x | x |
| *Mycobacterium tuberculosis* | o | o | x | x | x | x | x | x | o | x | x | x | x | x | x | x |
| *Corynebacterium glutamicum* | x | x | x | x | x | x | x | o | o | x | x | x | x | x | x | o |
| *Corynebacterium efficiens* | x | x | x | x | x | x | x | x | o | x | x | x | x | x | x | o |
| *Streptomyces coelicolor* | x | o | x | x | x | x | x | x | o | x | x | o | x | x | x | o |
| *Chlamydia trachomatis* | x | x | x | x | x | x | x | x | o | x | x | o | o | x | x | o |
| *Chlamydophila pneumoniae* | x | x | x | x | x | x | x | x | o | x | x | o | o | x | x | o |
| *Borrelia burgdorferi* | x | x | x | x | x | x | x | x | o | x | x | x | x | x | x | o |
| *Flavobacterium johnsoniae* | x | x | x | x | x | o | x | x | o | x | x | x | o | x | x | o |
| *Flavobacterium psychrophilum* | x | x | x | x | x | x | x | x | o | x | x | x | o | x | x | o |
| *Synechocystis sp* | x | o | x | x | x | x | x | x | o | x | x | x | x | x | x | o |
| *Chlorobaculum tepidum* | o | o | x | o | x | x | x | x | x | o | x | x | o | o | x | o |
| *Chlorobium chlorochromatii* | o | o | x | o | x | x | x | x | o | o | x | x | o | o | x | o |
| *Deinococcus radiodurans* | x | x | x | x | x | x | x | x | o | x | x | x | x | x | x | o |
| *Thermotoga maritime* | x | x | x | x | x | x | x | x | o | x | x | x | x | x | x | o |
| *Archaeoglobus fulgidus* | x | x | x | x | x | x | x | x | o | x | x | x | x | x | x | o |

| Bacteria[a] | *gmhB* | *lpcA* | *rfaC* | *rfaD* | *rfaE* | *rfaF* | *rfaG* | *ydcL* | *yfgL* | *ynbE* | *nlpI* | *ycdO* | *pal* | *tolQ* | *tolR* | *yciM* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *Pyrococcus horikoshii* | x | x | x | x | x | x | x | x | o | x | x | x | x | x | x | o |
| *Methylococcus capsulatus* | o | o | x | x | o | x | x | x | o | x | x | x | o | o | o | o |
| *Methanococcus jannaschii* | x | o | x | x | x | x | x | x | x | x | x | x | x | x | x | o |
| *Methanobacterium thermoautotrophicum* | x | x | x | x | x | x | x | x | o | x | x | x | x | x | x | o |
| *Halobacterium sp* | x | x | x | x | x | x | x | x | o | x | x | x | x | x | x | o |
| *Thermoanaerobacter tengcongensis* | x | o | x | x | x | x | x | x | o | x | x | x | x | x | x | o |
| *Thermodesulfovibrio yellowstonii* | x | o | o | o | x | x | o | x | o | x | o | o | o | o | o | o |
| *Thermanaerovibrio acidaminovorans* | o | o | o | x | o | x | x | x | x | x | x | o | o | o | o | o |

[a]Bacteria shown here are representatives of species of which genomic sequences are available in databases.
"o" and "x" represent the presence and absence of themotorelant genes in group B, respectively.

### 3.3.5 Expressional change caused by heat shock at CHT

None of the thermotolerant genes identified in this study were found to encode HSPs previously identified in *E. coli* except for *dnaJ*, *dnaK*, *degP* and *dnaQ*. To examine whether the thermotolerant genes were up-regulated at CHT or not, we examined transient change in expression of the genomic genes at CHT by DNA chip analysis. The results showed that 42 genes and 111 genes were significantly up-regulated and down-regulated, respectively (Table 2.6). The up-regulated genes were classified mainly into genes involved in the cellular process, and the down-regulated genes were classified into genes involved in energy metabolism, transport/binding protein and translation. However, none of the thermotolerant genes including *degP* and *dnaQ* as a heat-shock gene were identified as up-regulated genes except for *dnaJ* and *dnaK*. Taken together with data shown above, it is possible that the chaperone systems except for DnaJ/DnaK and GroEL/GroES are not neccesarily involved in thermotolerant mechanisms acquired at CHT. Therefore, it is likely that most products of thermotolerant genes are not HSPs and that the organism possesses a specific set of genes required for survival at CHT.

It is possible that some of the essential genes are crucial for growth at CHT. Such genes, however, could not be examined in this analysis because no disrupted mutants for these genes are available other than the conditional mutants. We thus listed essential genes with significant fluctuation in expression at CHT (Table 2.7). *groEL* (*groL*) encoding HSP was up-regulated, indicating the possibility that the gene product contributes to survival at CHT. Consistently, it was reported that GroEL appears as a mediator of evolution of extremely heat-resistant *E. coli* cells [Rudolph et al., 2010]. On the other hand, 90% of the down-regulated genes were mapped into the translation pathway (Figure S5), encoding for components of ribosomal proteins. It is thus possible that down-regulation of ribosomal genes is one of the strategies for survival at CHT in *E. coli*. Noteworthily, Alix *et al.* reported that ribosome biogenesis in *E. coli* is high temperature-sensitive and DnaK-dependant and predicted that high temperature causes a severe limitation in DnaK/DnaJ to hamper ribosome assembly because heat-induced misfolded proteins would titrate out all the free DnaK/DnaJ [Al Refaii and Alix, 2009; René and Alix, 2010].

**Table 2.6** Genes significantly up-regulated and down-regulated at CHT.

| Classification | Number of classified genes | Up-regulated | Down-regulated |
|---|---|---|---|
| 1) Amino acid metabolism | 136 (5) | 0 (0) | 3 (0) |
| 2) Biosynthesis of cofactors, prosthetic groups, carriers | 127 (28) | 1(0) | 0 (0) |
| 3) Cell envelope | 171 (16) | 0 (0) | 5 (0) |
| 4) Cellular process | 102 (63) | 6 (1) | 5 (0) |
| 5) Central intermediately metabolism | 153 (8) | 2 (0) | 8 (0) |
| 6) Energy metabolism | 358 (7) | 2 (0) | 22 (0) |
| 7) Fatty acid/Phospholipid metabolism | 40 (22) | 0 (0) | 2 (1) |
| 8) Nucleotide metabolism | 113 (0) | 0 (0) | 4 (0) |
| 9) Regulatory functions | 107 (1) | 1 (0) | 4 (0) |
| 10) Replication | 79 (23) | 0 (0) | 1 (0) |
| 11) Transport/binding protein | 76 (1) | 0 (0) | 12 (0) |
| 12) Translation | 354 (101) | 4 (1) | 23 (20) |
| 13) Transcription | 77 (13) | 1 (0) | 1(1) |
| 14) Other categories | 336 (14) | 3 (0) | 5 (0) |
| 15) Hypothetical | 2269 (10) | 20 (0) | 16 (0) |
| Total number of genes | 4498 (312) | 42 (2) | 111 (22) |

*Genes with expression ratios of more than twofold, or less than 25%, are shown as significantly up-regulated and down-regulated, respectively.

*Digits inside parentheses represent the number of essential genes.

**Table 2.7** Essential genes significantly up-regulated and down-regulated at CHT.

| Classification[a] | Pathway[a] | Gene |
|---|---|---|
| Up-regulated | | |
| Cpn60 chaperonine | RNA degradation | *groEL* |
| tRNA-Leu | Transfer RNA | *leuU* |
| Down-regulated | | |
| Lipid metabolism | Fatty acid biosynthesis | *fabG* |
| Transcription | RNA polymerase | *rpoA* |
| Translation | 16S rRNA processing protein | *rim* |
| Translation | Translation factors | *fusA* |
| Translation | Ribosome | *rpsP, rplQ, rpsD, rpsK, rpsM, rpmC, rplP, rpsC, rplV, rpsS, rplB, rplW, rplD, rplC, rpsJ, rplJ, rplL, rpsR* |

[a]Classification and Pathway accoding to the KEGG PATHWAY are shown. (http://www.genome.jp/kegg-bin/show_organism?menu_type=genome_info&org=ecj).

### 2.3.6 Further consideration on mechanisms for survival at CHT

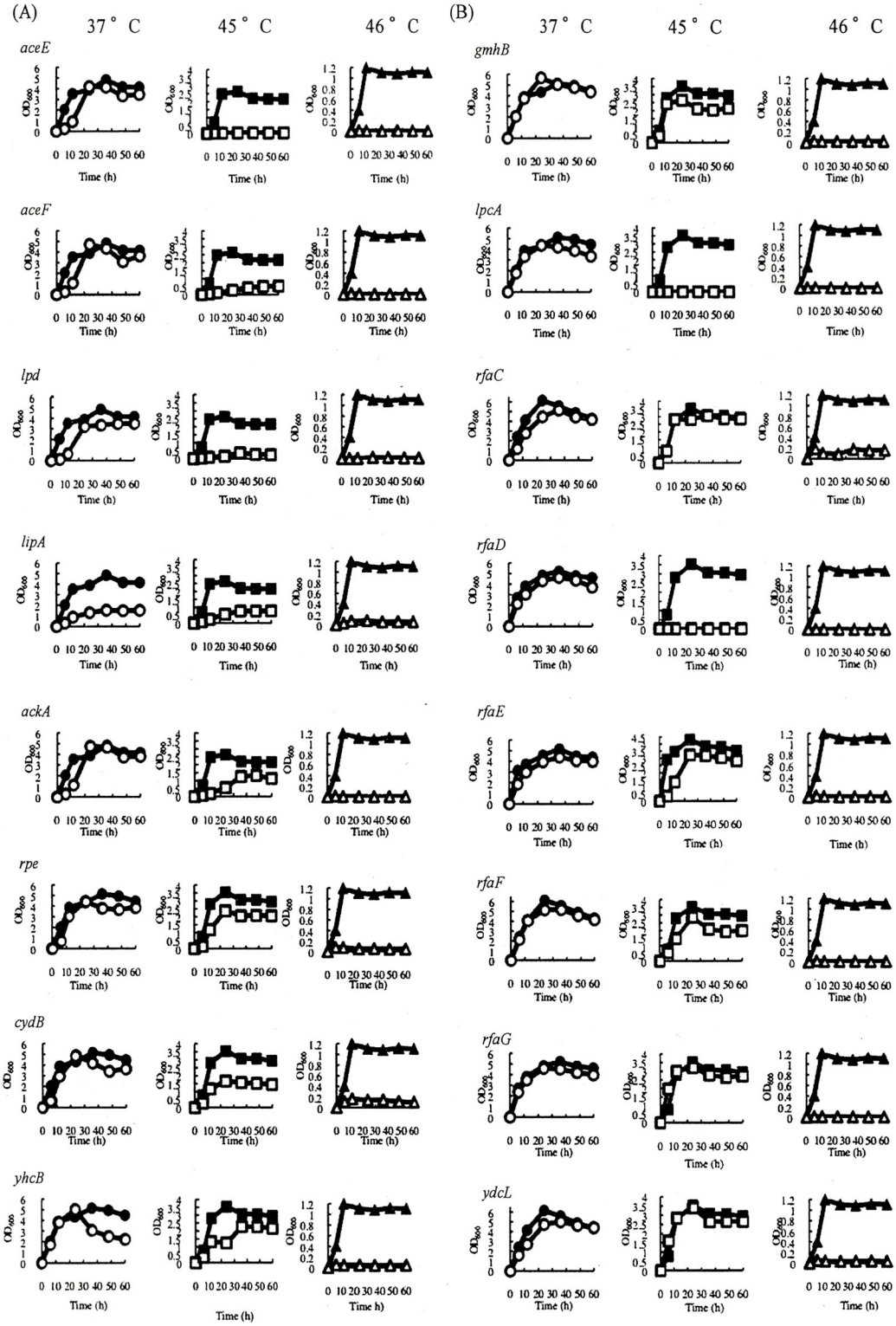
Two groups of DNA double-strand repair and chaperone/proteinase genes may contribute to endurance against oxidative stress in addition to CHT. Evidence that a higher temperature results in accumulation of more oxidative stress [Noor et al., 2009] and the finding that mutants of all members in both groups exhibited sensitivity to oxidative stress allow us to speculate that oxidative stress is a main cause of DNA double-strand breaks and of damage to proteins at CHT. Interestingly, oxidative stress is involved in heat-induced cell death in *S. cerevisiae* [Davidson et al., 1996], which is supported by the findings that overexpression of catalase and superoxide dismutase genes could increase the degree of thermotolerance and that the thermotolerance is

increased under anaerobic conditions. We thus assume that CHT somehow causes intracellular oxidative stress to elicit harmful effects on cells as a secondary stress.

Significant suppression of the thermosensitive phenotype by a defect in the group of energy metabolism (Group A) by the addition of glucose suggests the limitation of energy level at CHT in the organism. The limitation seems to be resolved by alternative pathways that may generate ATP by glucose assimilation. The requirement of ATP at CHT may be consistent with expression of ribosomal genes. Many genes for ribosomal proteins were found to be down-regulated by exposure to CHT, and the disrupted mutant of *dksA* that encodes a negative regulator for rRNA genes became thermosensitive to CHT. These findings and evidence that translation as a ribosomal activity utilizes much energy, up to about 90% of energy consumed in cells [Lehninger and Cov, 1993], suggest that cells manage to reduce energy consumption under a severe condition at CHT. Such saved energy would be utilized for other crucial activities such as repair or degradation of damaged DNA or protein molecules. A smooth translational process at CHT might also save energy, for which S6 and L36 of ribosomal proteins in addition to SmpB may have important functions.

Several strategies for *E. coli* to survive at CHT were discovered. Most of them may also be responsible for other stresses and are conserved even in mesophilic bacteria. Early glycosyltransferases for LPS core biosynthesis for proper outer membrane assembly and permeability barrier function and the sulfur-relay system for tRNA modification might have been acquired for the organism to perform a main task to survive at CHT. Considering the genetic conversion of non-thermotolerant to thermotolerant bacteria, the two strategies might be applicable.

**Suppement Information**


(A)
37 ° C
45 ° C
46 ° C
aceE
aceF
lpd
lipA
ackA
rpe
cydB
yhcB
(B)
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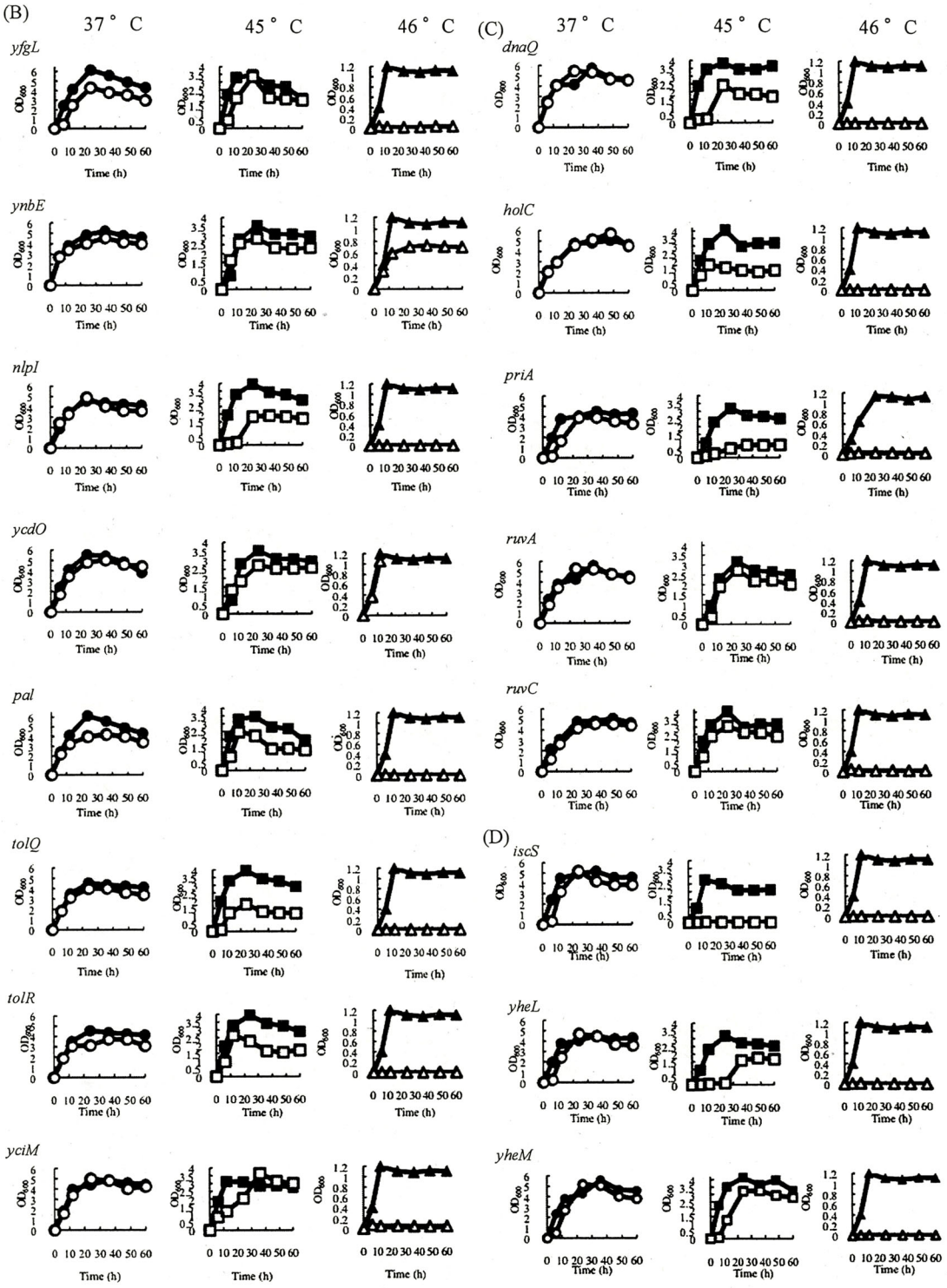
lpcA
rfaC
rfaD
rfaE
rfaF
rfaG
ydcL
Time (h)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nlpI
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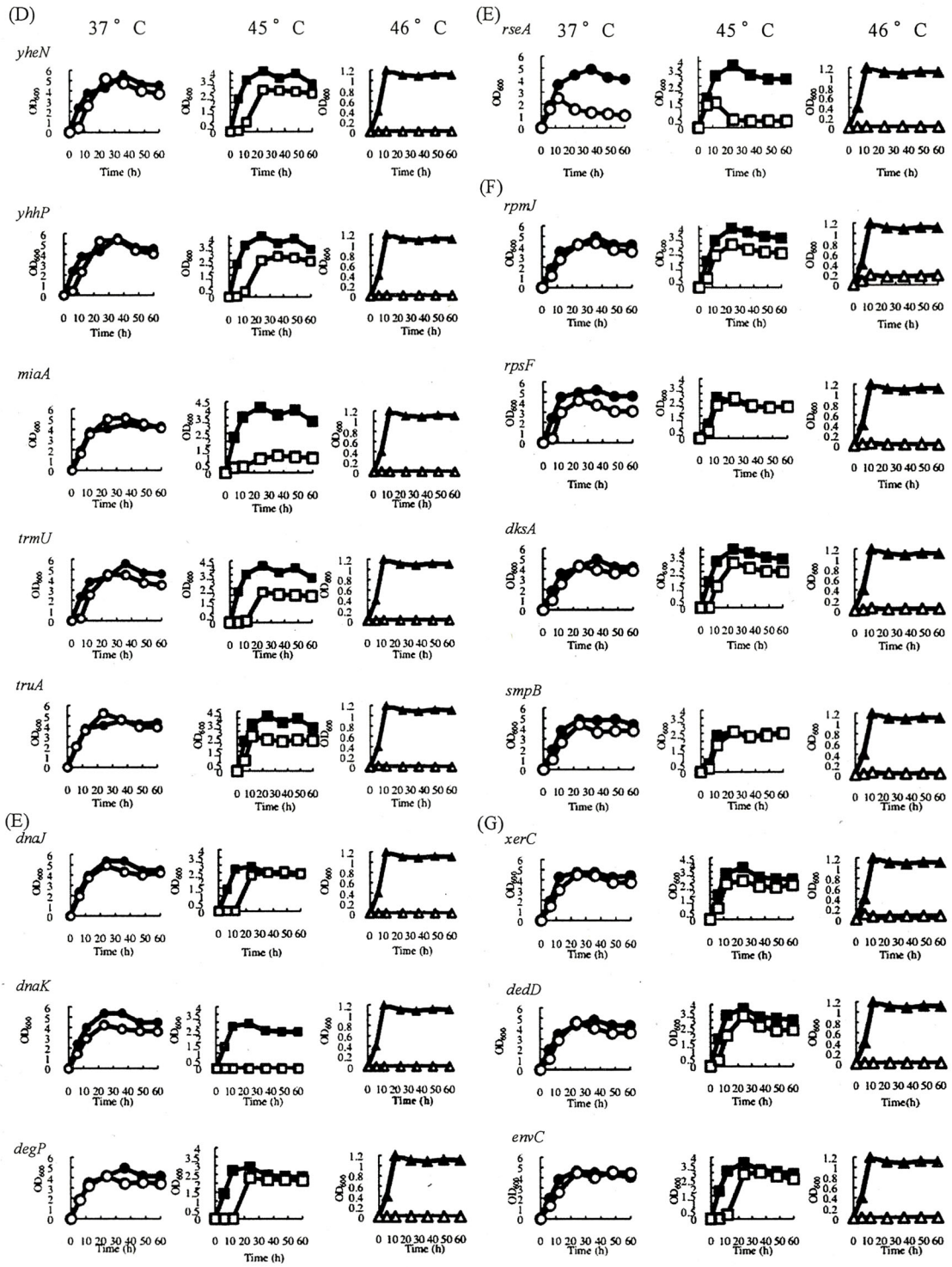
pal
tolQ
tolR
yciM
dnaQ
holC
priA
ruvA
ruvC
(D)
iscS
yheL
yheM
OD600
Time (h)
0 10 20 30 40 50 60


(D)
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45 ° C
46 ° C
yheN
yhhP
miaA
trmU
truA
(E)
dnaJ
dnaK
degP
(E)
rseA
(F)
rpmJ
rpsF
dksA
smpB
(G)
xerC
dedD
envC
OD600
Time (h)
0 10 20 30 40 50 60

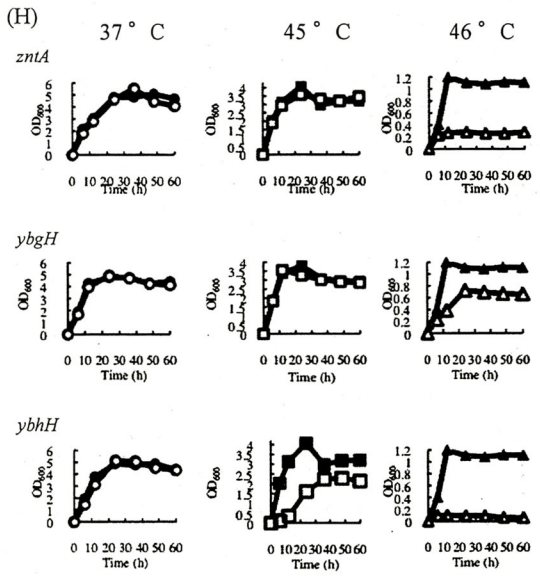


**Figure S1 Growth of thermosensitive mutants in LB liquid culture at different temperatures.** Each 51 thermosensitive mutant strain (opened symbols) and the parental strain, BW25113 (closed symbols), were grown in 30 ml LB medium at 37 °C (circles), 45 °C (squares), or 46 °C (triangles). At the times indicated, turbidity at $OD_{600}$ was measured. A, group A; B, group B; C, group C; D, group D; E, group E; F, group F; G, group G; H, others.

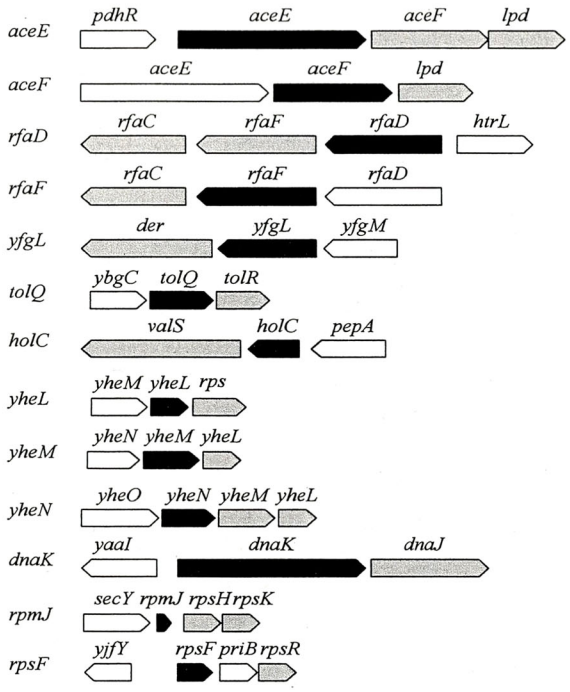


**Figure S2 Gene organizations around genes having either an essential gene or a thermotolerant gene as a just downstream gene.** Gene organizations around 13 thermotolerant genes that have either an essential gene or a thermotolerant gene as a just downstream gene are depicted. Black boxes represent identified 13 thermotolerant genes. Grey boxes represent essential or thermotolerant genes. The direction of boxes shows the direction of transcription.

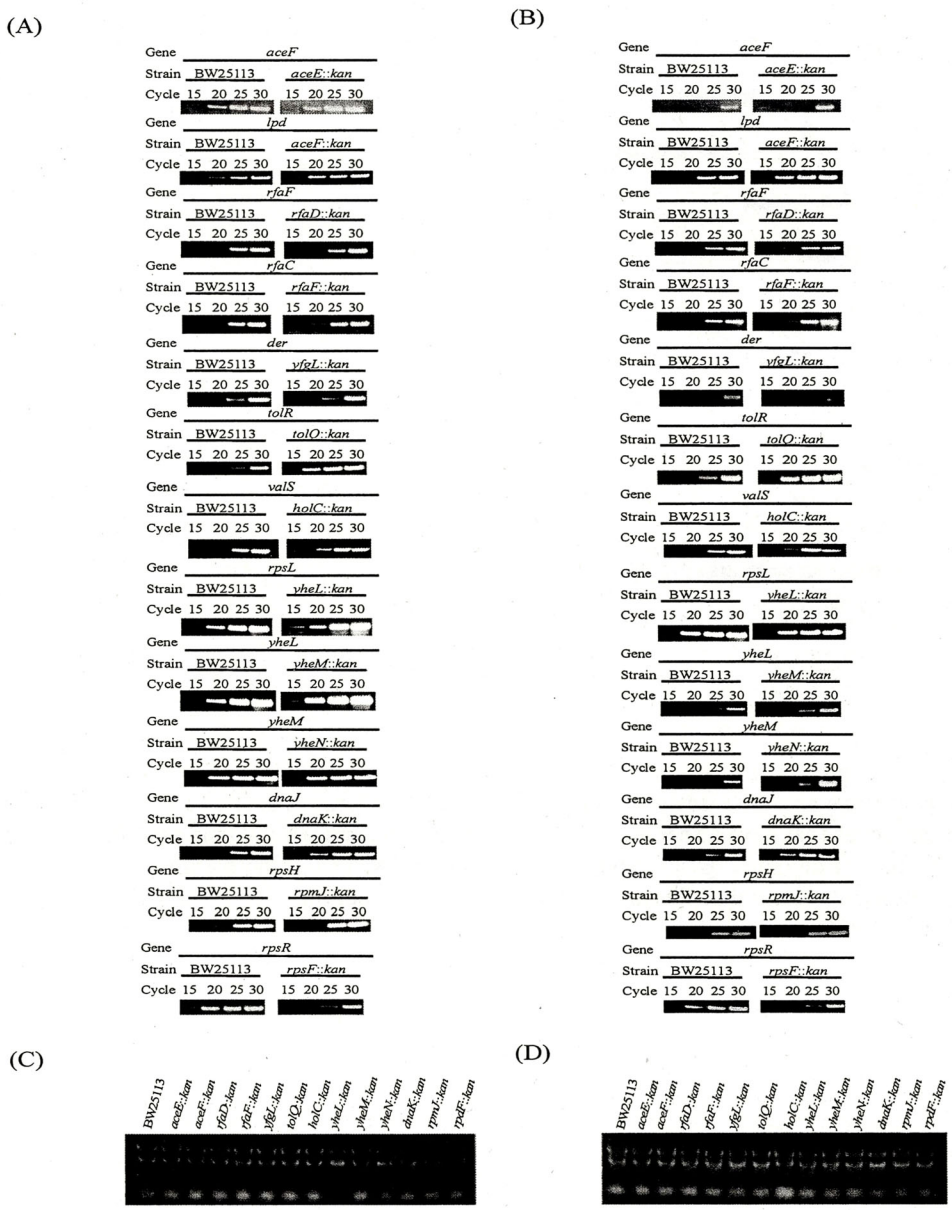


**Figure S3 Testing of possible polar effects by the *aph* insertion.** Total RNA was prepared from cells cultured at 37°C (A, C) and 47°C (B, D) as described in Materials and methods. RT-PCR was performed with primers specific for a just downstream gene of each thermotolerant gene to amplify about 500-bp DNA fragments. (A and B) After RT reaction, PCR was performed 15, 20, 25 and 30 cycles and the products were analyzed. (C and D) As a control, each total RNA (10 μg) was submitted to 1.2% agarose gel electrophoresis and staining with ethidium bromide.

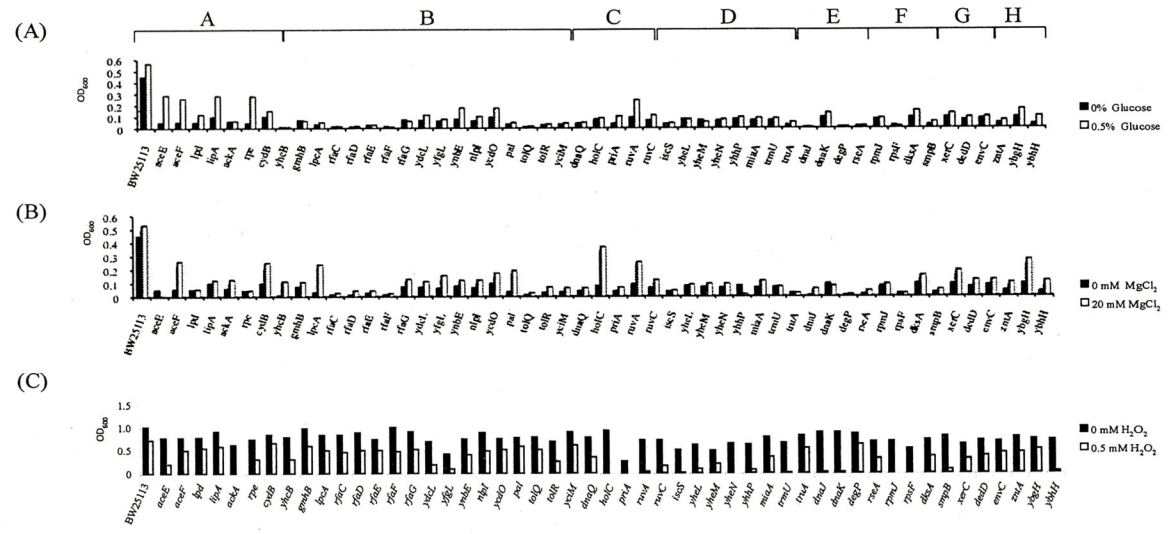


**Figure S4 Effects of addition of glucose and $MgCl_2$ and sensitivity to $H_2O_2$.**
Thermosensitive mutant strains are shown by gene names. Growth conditions are described in Materials and methods. Black and white columns represent turbidity under the conditions with or without supplements (0.5% glucose (A) or 20 mM $MgCl_2$ (B)) or 0.5 mM $H_2O_2$ (C).

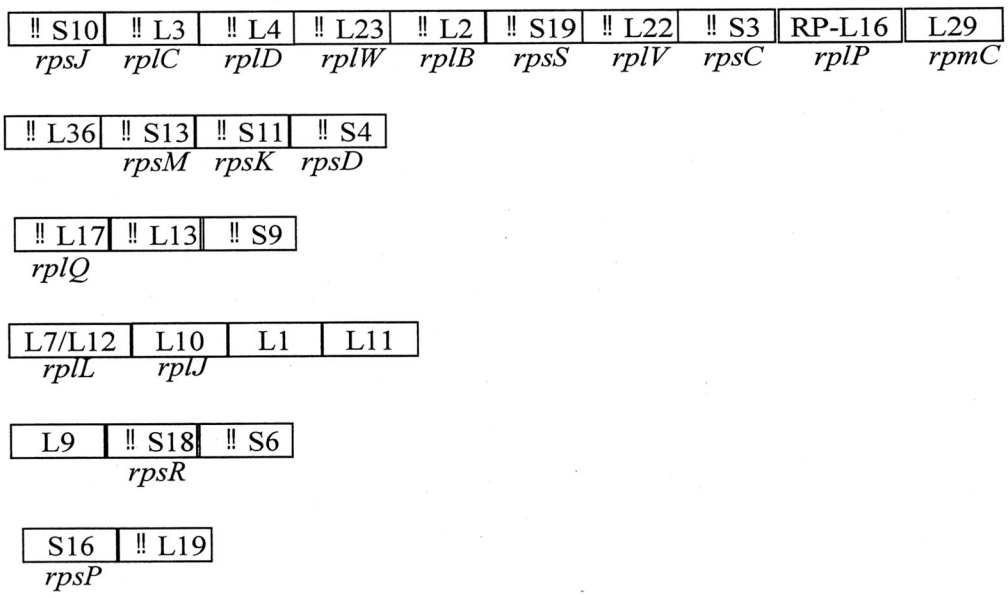


**Figure S5 Down-regulated genes for ribosomal proteins.** Systematic analysis of gene function was performed with a database of KEGG PATHWAY. Down-regulated genes for ribosomal proteins were mapped into 6 operons.

# Chapter 3

## Molecular metabolism of thermotolerance in ethanologenic themotolerant *Zymomonas mobilis* applicable for high temperature fermentation


### Astract

To identify thermotolerant genes responsible for growth of the ethanologenic thermotolerant *Z. mobilis* at critical high temperature (CHT), TISTR 548 strain that was chosen as a thermotolerant strain was subjected to transposon mutagenesis via transconjugation with a mobilizable plasmid harboring the transposable element (Tn*10*) and growth experiment at 39°C, CHT for the strain. Among about 4,000 of the transconjugants obtained, 42 mutants that were found to be dramatically defective in growth at the CHT, which were selected as thermosensitive mutant strains. The insertion site of Tn*10* within the genome was then determined by thermal asymmetric interlaced-(TAIL) PCR followed by DNA sequencing. As a result, 17 genes related to the thermotolerance have been identified. Some of these were related to membrane biosynthesis and lipid metabolism, recombination for DNA repair and replication, tRNA modification, transportation system, which may have a direct or in direct relation to the thermotolerance mechanism. Interestingly, the results also revealed a partial overlapping between genes required for the thernotolerance and those for tolerance to other stresses. Our findings provide molecular mechanisms underlying a survival of *Z. mobilis* at CHT which may advantages in production of ethanol and other useful materials at high temperature.


### 3.1 Introduction

*Z. mobilis* as a Gram-negative, non-spore forming and polar flagellated bacterium has been isolated from sugar cane as well as alcoholic beverages such as African palm wine and is known to cause cider sickness and spoiling of beer [Swings and De Ley, 1977]. Gibbs and DeMoss [1954] discovered its anaerobic catabolism of glucose following the discovery of the Entner-Doudoroff (ED) pathway in the early 1950s. The ED pathway utilizes 1 mol of glucose to yield 2 mol of pyruvate, which are

then decarboxylated to acetaldehyde and reduced to ethanol, which was lower than that of the Emden-Meyerhof pathway of the conventional ethanol producer yeast [Panesar et al., 2006] and is generally recognized as safe (GRAS) status [Yang et al., 2010]. The world wide interest have thus focused on the application of this organism as an efficient microorganism for production of the bio-fuel [Osman and Ingram, 1987; Ingram et al., 1998; Altintas et al., 2006; Cazetta et al., 2007; Bai et al., 2008; Agrawal et al., 2011] and of useful materials such as oligosaccharide; lactosucrose [Han et al., 2009] and levan [Chiang et al., 2009] that can be applied for food and pharmaceutical industries [Calazans et al., 1997] and medicine [Yoo et al., 2004].

*Z. mobilis* TISTR 548 is one of thermotolerant strains [Sootsuwan et al., 2008] isolated from Thailand, and can grow at 39°C [Charoensuk et al., 2011], which is about 10°C over the reported optimum temperature of *Z. mobilis* [Swing and De Ley, 1977]. The definition of thermotolerance is that a mesophile is able to grow at about 10°C to 15°C higher than the general same species [Saeki et al., 1997].

There are a number of advantages in application of thermophilic microorganisms for production of ethanol and useful materials: reduction in cooling cost, higher saccharification and fermentation rates, continuous ethanol removal and reduced contamination [Singh et al., 1998]. However, in even thermotolerant strains, heat stress may impact on their growth viability [Basso et al., 2008;Babiker et al., 2010;] and also they may cause stuck fermentations, especially when other fermentation factors reach critical values, i.e. low pH, high ethanol content, high osmolarity, nutrient supply and temperature etc. [Piper, 1995; Carmelo, 1998; Ciani, 2006; Pizarro, 2007; Coleman et al., 2007; Gibson et al., 2007].

When exposed to environmental stress, cells alter their transcriptional program, resulting in the immediate down-regulation of housekeeping genes and dramatic increase of expression of a set of crucial defense and/or adaptation genes which are essential for cell viability [Ruiz-Roig et al., 2010], and under more sever situation, protein denaturation, transient cell cycle arrest and variations in membrane fluidity and structure occur [Benschoter and Ingram, 1986]. Cells can adapt to relatively mild (not sever) stress, which is known as the heat stress response [Riezman, 2004; van der Veen et al., 2007] to protect their components [Guyot et al., 2005]. However, mechanisms underlying heat tolerance may be complicated in which many different genes are assumed to be involved but the exact mechanism has not been fully delineated [Jeffries and Jin, 2000].

Since the ethanol fermentation process is exothermic [Uden, 1981; Ghose, 2004], ethanologenic microorganisms seem to be exposed to heat stress in addition to other

stresses including ethanol [Attfield, 1997; Wei et al., 2007]. The effect of heat and ethanol stresses on the protein expression pattern in *Z. mobilis* is evident in several polypeptides that associated with the cell envelope fraction in addition to specific heat shock proteins [Michel and Starka, 1986; Thanonkeo 2007]. Some of them would be involved in heat and ethanol tolerance mechanisms in the organism. Only limited information is available on the capacity of the thermotolerant *Z. mobilis* to grow at high temperatures. The molecular mechanisms that allow *Z. mobilis* cells to survive at high temperature environments are very useful for high-temperature fermentation of bioethanol and other useful materials.

This study aims to identify the thermotolerant mechanism supporting the growth of thermotolerant *Z. mobilis* at high temperature. Comparison of two TISTR strains in Thailand, ZM4, NCIMB 11163 and LMG revealed that TISTR 548 was the most thermotolerant. TISTR 548 was then subjected to transposon mutagenesis, via transconjugation with a mobilizable plasmid harboring the transposable element (Tn*10*) and thermosensitive mutants were isolated. The mutation site of each mutant was determined by thermal asymmetric interlaced-(TAIL) PCR followed by DNA sequencing and thermotolerant genes were identified. These genes were then categorized and compared with those in *E. coli* shown in the 2nd Chapter.

## 3.2 Materials and methods

### 3.2.1 Materials

A DNA sequencing Kit (ABI PRISM ® Terminator v 3.1 Cycle sequencing Kit) was obtained from Applied Biosystem Japan. Oligonucleotide primers were synthesized by Proligo Japan K.K. (Tokyo, Japan). Other chemicals were all of analytical grade and obtained from commercial sources.

### 3.2.2 Microorganisms

*Z. mobilis* strains used in this study were grown in YPD medium consisting of 3 g of glucose, 0.5 g of peptone and 0.3 g of yeast extract in 100 ml of IEW. Cells cultured for 9 h and 24 h, these terms correspond to optical densities of about 0.8 and 2.5 at 550 nm under a shaking condition and about 1.5 and 3.0 at 550 nm under a static condition, respectively, were used as exponential- and stationary-phase samples, respectively. *E. coli* S17-1 carrying plasmids of the pSUP2021 Tn*10* was grown in LB

medium supplemented with 12.5 μg/ml of tetracycline and *Z. mobilis* transposon-inserted mutants were grown in YPD medium containing 12.5 μg/ml of tetracycline.

### 3.2.3 Growth and ethanol production of *Z. mobilis* strains

*Z. mobilis* TISTR 548 and 405 reported as the thermotolerant strain [Sootsuwan et al., 2008; Charoensuk et al., 2011] were compared in growth with the other type strains of ZM4 strain [Seo et al., 2005], NCIMB 11163 [Kouvelis et al., 2009] and LMG. Cells were grow on YP containing 3% glucose at 30°C for 18 h, and then diluted cells to ten times by YPD medium. Five μl of each serial diluted cell was spotted onto an YPD agar plate with or without supplemented agents, and incubated at 30°C or 37°C. Their growth was observed and their pictures were taken after 48 h incubation. Growth and ethanol production in YPD medium were carried out in triplicate for each strain.

### 3.2.4 Conjugation

*E. coli* S17-1 carrying pSUP2021 Tn*10* as a donor strain for conjugal mating was routinely grown in LB medium containing 12.5 μg/ml of tetracycline under 100 rpm-shaking condition at 37°C. The recipient *Z. mobilis* TISTR 548 was grown in YPD medium at 30°C under a static condition. After both strains were grown to mid-log phase, about 0.8 of $OD_{600}$ and washed three times with LB broth medium and each bacterium was recovered by centrifugtion for 1 min at 5,000 rpm then mixed at a ratio of donor/recipient of 3:2, incubated for 3 h at 30°C to allow their mating and further incubated at 30°C for 5 h on the surface of LB agar plates with 15 μl dot spot of suspension of the mixed donor and recipient cells. After mating, the cells were recovered and re-suspended in YPD medium, and then spread on YPD agar plates containing 0.2% of acetic acid and 12.5 μg/ml tetracycline HCl. Tn*10*-inserted mutants appeared after 3 days at 30°C, which were subsequently screened for further experiments.

### 3.2.5 Screening of thermosensitive mutant strains

The first screening was performed on YPD agar plates. Among about 4,000 transconjugants obtained were grown at 30°C and 39°C, mutant strains that could not grow or poorly grew on the plates were selected for the 2$^{nd}$ screening. The second

screening was performed in 1 ml of YPD medium at 30°C and 39°C for 24 h under a static condition with the initial inoculation at 0.05 of $OD_{550}$. The mutant strains that gave the $OD_{550}$ less than a half of that of the parent strain were selected for the final screening, which was performed in 30 ml of YPD medium under the same condition of the 2nd screening. The mutant strains that gave the $OD_{550}$ in the significant reduction compared to that of the parent strain were selected and defined as thermosensitive mutants.

### 3.2.6 Effect of various stresses on growth of thermosensitive mutants

To determine the overlapping effect between high temperature and other stresses, thermosensitive mutants were subjected to osmotic, ethanol, oxidative and antibiotic stress. Growth ability of thermosensitive mutants was determined on YPD agar plates supplemented with 12% (w/v) of glucose, 2.5% (v/v) of ethanol or 2 mM $H_2O_2$. Cells grown in YPD medium at 30°C for 18h under a static condition were serially diluted with YPD medium, and 5 µl of each diluted sample was spotted on YPD agar plates with or without supplemented agents and incubated at 30°C for 48 h. Growth ability test was carried out in duplicate for each strain.

### 3.2.7 DNA manipulation

Genomic DNA isolation and other general molecular biology techniques were performed as described by Sambrook et al [30Sambrook et al., 1989]. Concentration of isolated genomic DNA was measured by using Nanodrop (Nanodrop Technologies, Wilmington, DE).

### 3.2.8 Identification of transposon (Tn*10*)-inserted site in the genome of thermosensitive mutants by thermal asymmetric interlaced (TAIL)-PCR and nucleotide sequencing

Since transposon (Tn*10*) was randomly inserted into the genome in this experiment, the unknown site inserted of Tn*10* was determined by TAIL-PCR as reported previously [Lui et al., 1995] with several modifications as described below. Three serials of PCR were performed on Mycycler™ thermal cycler (Bio-Rad) in different conditions (Table 3.1) with specific primers, TnISR-1

(GATCCTCTCGTTTGTTGCGGTCAGGCC) [Deeraksa et al., 2005], TnISR-1.5 (AGGGCTGCTAAAGGAAGCGG) and TnISR (ACGAAGCGCAAAGAGGAAGCAGG) together with degenerated primer, AD2 (GTNCGASWCANAWGTT) [Lui et al., 1995]. The first PCR was carried out in a 50 µl of mixture containing 10 ng of chromosomal DNA, 5.0 µM of specific primer TnISR1, 25 µM of AD2 primer, 500 µM of each dNTP, 0.5 U PrimSTAR (Takara), a buffer supplied for enzyme and sterilized distilled water. The product of 1st PCR was 50-times diluted and used as a template for the 2nd PCR in the same reaction mixture as the 1st PCR except that the TnISR1.5 was used as a specific primer. The 3rd PCR was similarly performed as the 2nd PCR with a specific primer TnISR2 and 12.5 µM of AD2. The products from 2nd or 3rd PCR were purified by using a column (Takara) and used as a template for the reaction of nucleotide sequencing. The product of sequencing reaction was then subjected to sequence analysis using an ABI PRISM 310 (PE Biosystems).

**Table 3.1** Reaction parameters of TAIL-PCR for amplification of the franking region of Tn*10* inserted in the thermosensitive mutant strains of *Z. moobilis* TISTR 548 with the modified from previous report (Liu and Whitter, 1995).

| Reaction Primer combination | Steps | Number of cycles | Cycle (supercycle) parameter |
|---|---|---|---|
| 1st PCR | 1 | 1 | 93°C, 1 min; 95°C, 1 min |
| (TnISR-1/AD2) | 2 | 5 | 94°C, 30 sec; 64°C, 1 min; 72 °C, 2 min |
| | 3 | 1 | 94°C, 30 sec; 25°C, 1 min; ramping to 72 °C, 3 min; then 72°C, 2 min |
| | 4 | 15 | 94°C, 30 sec; 64°C, 1 min; 72 °C, 2 min; 94°C, 30 sec; 64°C, 1 min; 72°C, 2 min; 94 °C, 30 sec; 44°C, 1 min; 2 min; 72 °C, 2 min |
| 2nd PCR (TnISR-1.5/AD2) | 1 | 15 | 94°C, 30 sec; 64°C, 1 min; 72 °C, 2.5 min; 94°C, 30 sec; 64°C, 1 min; 72°C, 2.5 min; 94 °C, 30 sec; 44°C, 1 min; 2 min; 72 °C, 2.5 min |
| 3rd PCR | 1 | 1 | 94 °C, 1 min; |
| (TnISR-2/AD2) | 2 | 30 | 94 °C, 30 sec; 50°C, 1 min; 72 °C, 2.5 min |

### 3.2.9 Comparison of nucleotide sequences of flanking regions of Tn*10* inserted with those in database

Nucleotide sequences of flanking regions of Tn*10* inserted in each thermosensitive mutant were compared with those in the DDBJ database by using BLAST [Altschul et al., 1990]. Further comparison and alignment of nucleotide sequences were conducted by using GENETYX (Software Development, Tokyo, Japan). The phylogenetic tree was constructed using CLASTAL W (DDBJ).

### 3.2.10 Analytical procedures

Ethanol and glucose concentrations in the medium were measured by high performance liquid chromatography (HPLC) (Hitachi Chemical, Japan) using a Gelpack GL-C610-S column (7.8 x 300 mm). A medium fraction was prepared as a supernatant after a low-speed centrifugation of cultures at different cultivation time points and diluted 10 times before applying to HPLC. Using HPLC with a reflective index detector, chromatography was performed at 60°C and degassed water was used as a mobile phase at a flow rate of 0.3 ml/min. Concentrations of ethanol and glucose were determined by comparing a detected area with that of the corresponding standard sample.

### 3.2.11 Statistics

Unless otherwise indicated, mean and standard deviations (SD) were calculated from data of at least three independent experiments. The variations between the experiments were estimated by SD, and the statistical significance of changes was estimated by two-sample *t*-test assuming equal variances. ***, ** and * mean $p$ <0.001, <0.01 and <0.05, respectively. The level of significance was accepted at $p \leq 0.05$.

## 3.3 Results

### 3.3.1 Comparative growth and ethanol production of thermotolerant *Z. mobilis* with the other type strains

*Z. mobilis* TISTR 548 and 405 as a thermotolerant strain [Sootsuwan et al., 2008; Charoensuk et al., 2011] was compared in growth and ethanol production with other thermosensitive type strains of ZM4 [Seo et al., 2005] and NCIMB 11163

[Kouvelis et al., 2009] at high temperature. A phylogenetic tree (Figure 3.1) with 16S rDNA revealed that the two thermotolerant strains were closely related to the two thermosensitive strains.

The growth ability of cells was examined in YPD medium containing 3% (w/v) glucose with or without 2.5% v/v ethanol or 3 mM $H_2O_2$ and in YPD medium containing 12% (w/v) glucose at high temperature after 48 h. As shown in Figure 3.2, only *Z. mobilis* TISTR 548 and 405 strains could grow well at high temperature. By the evaluation of the growth ability and the ethanol productivity at high temperature (Figure 3.3), TISTR 548 was chosen for further experiments.

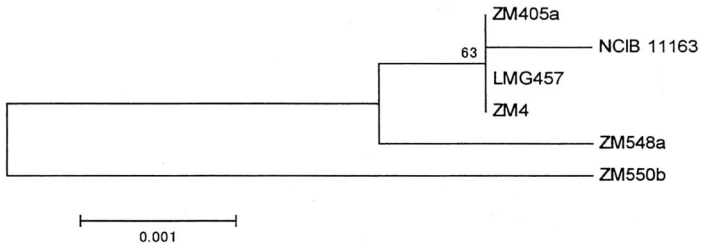


**Fig. 3.1** Phylogenic tree analysis of 16 s rRNA of the thermotolerant *Z. mobilis* TISTR 548 and other type strains.

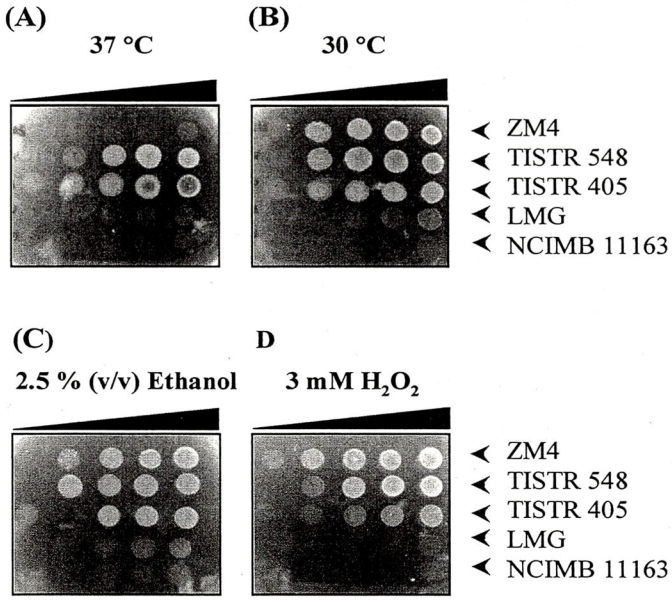


**Figure 3.2** Comparison of growth abilities of the *Z. mobilis* TISTR 548 with other type strains under stresses condition.
Growth ability of *Z. mobilis strains* on YPD agar plate; cells suspension of 18 h with serial dilution were spotted on the YPD containing 3% glucose agar plates and incubated at 30°C (A) and 37°C (B), and on the YPD containing 3% glucose agar plates supplemented with 2.5% v/v ethanol (C) or 3mM $H_2O_2$ (D) at 30°C. Photos shown are representatives.

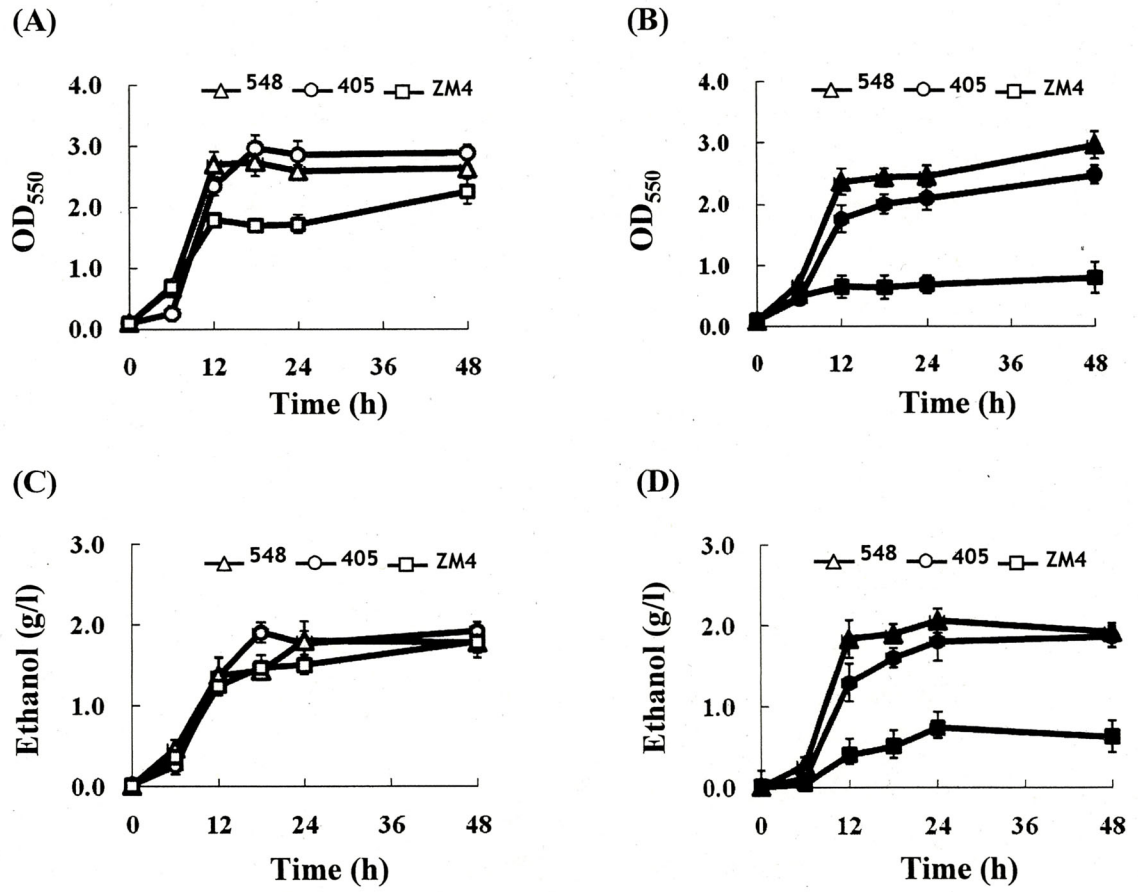


**Fig. 3.3** Growth of *Z. mobilis* strains in YPD medium under a static condition. Growth ability was compared in 30 ml of YPD containing 3 % glucose under a static condition at 30°C (A) and 39°C (B), and at the same time ethanol concentration at 30°C (C) and 39°C (D) was measured. Reported values are the mean (±SD) of three independent experiments. *p* values were calculated by two-sample *t*-test assuming equal variances.

### 3.3.2 Screening of thermosensitive mutants and identification of thermotolerant genes in *Z. mobilis*

To obtain thermosensitive mutants of *Z. mobilis* TISTR 548, a mobilizable plasmid hearing the transposable element (Tn*10*) was transferred from *Escherichia coli* S17.1 as a donor to *Z. mobilis* TISTR 548 as a recipient. About 4,000 transconjugants obtained were cultivated on YPD plates at 30°C or 39°C to observe a thermosensitive phenotype such an absence of growth or smaller colonies at high temperatures. As the result, 126 mutant strains were selected as candidates of thermosensitive mutants. The

growth phenotype of these mutants were further examined by measuring cell growth at 39°C, and finally 42 mutants with a strong defective growth were obtained, which exhibited significantly defective growth in YPD medium at 39°C but not at 30°C when compared with the parental strain (Figure 3.4).

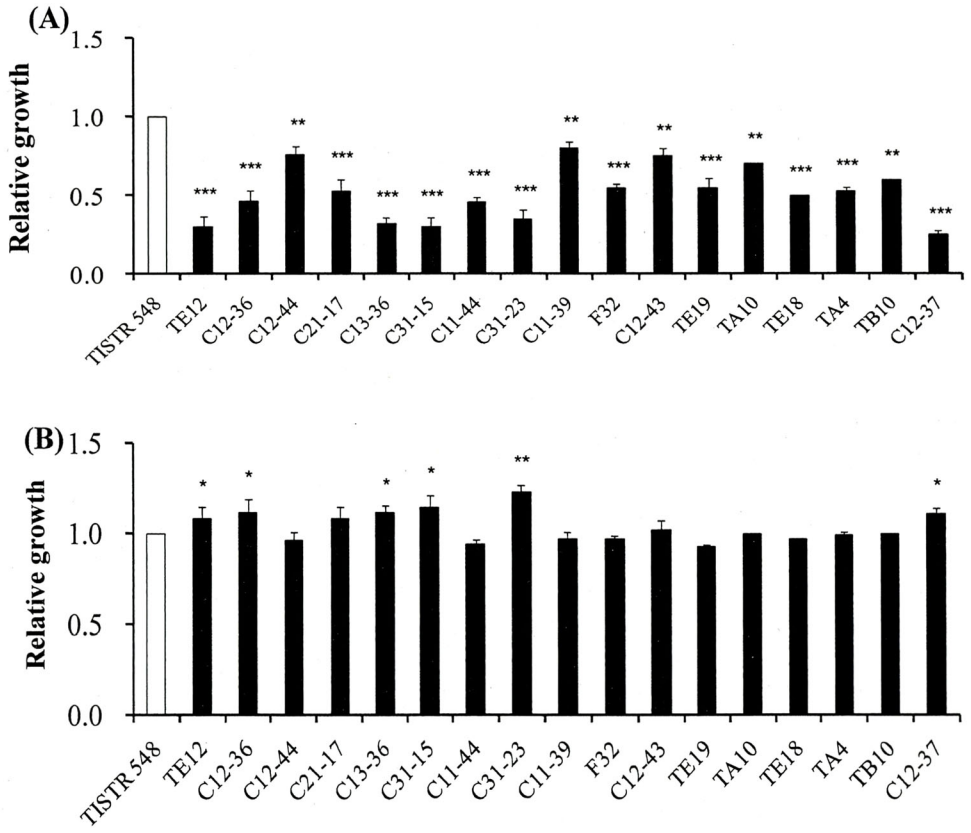


**Figure 3.4** Relative growths of thermosensitive mutant *Z. mobilis* strains with its parental strain. Cells were grown in YPD medium under static condition. The 0.05 initial $OD_{550\ nm}$ of inoculums were used to grown cells in 30 ml of YPD containing 3 % glucose under static condition. Growth at 30°C (A) and 39 °C (B), and ethanol production at 30°C (C) and 39 °C (D). Reported values are the mean (±SD) of three independent experiments. *p* values were calculated by two-sample *t*-test assuming equal variances.

**Table 3.2** Functional distribution of *Z. mobilis* thermotolerant genes

| Functional category | Organisms (encoded protein) | Identities (%) | Frequencies (No.) | Sensitivity to stresses[a] | | |
|---|---|---|---|---|---|---|
| | | | | Osmotic stress | Ethanol stress | Oxidative stress |
| (B) Membrane stabilization | | | | | | |
| TE12; Glucosamine/fructose 6-phosphate aminotransferase, isomerizing | *Z. mobolis* ZM4 (ZMO0056) | 98(95) | 1 | X | X | X |
| C12-36; Squalene hopene cyclase | *Z. mobolis* ZM4 (*shc*; ZMO1548) | 99(97) | 3 | S | S | X |
| C11-44 ; TolQ biopolymer transport proteins | *Z. mobolis* ZM4 (ZMO0161) | 99(98) | 1 | S | S | X |
| (C) DNA repair | | | | | | |
| CF-32 ; DNA repair protein | *Z. mobolis* ZM4 (*rad*C; ZMO0663) | 99(95) | 1 | X | X | X |
| C12-43; Exonuclease VII large subunit | *Z. mobolis* ZM4 (*xse*A; ZMO0300) | 99(97) | 1 | X | X | X |
| (D) tRNA modification | | | | | | |
| C12-44 ; rRNA methylase | *Z. mobolis* ZM4 (ZMO1328) | 99(98) | 1 | S | S | X |

| | | | | | | |
|---|---|---|---|---|---|---|
| (E) Chaperon/protease | | | | | | |
| C21-17 ; Predicted Zn dependent peptidase | *Z. mobolis* ZM4 (ZMO1422) | 98(97) | 1 | S | X | S |
| (F) Translation | | | | | | |
| C31-23 ; Preprotein translocase subunit | *Z. mobolis* ZM4 (ZMO1897) | 99(98) | 1 | X | X | X |
| (G) Cell division | | | | | | |
| TE19; ATPase involved in chromosome partitioning-like protein | *Z. mobolis* ZM4 (ZMO0236) | 98(96) | 1 | X | X | X |
| (H) Anti-oxidative stress | | | | | | |
| C11-39; Xanthine/Uracil permease family protein | *Z. mobolis* ZM4 (ZMO0969) | 99(97) | 21 | X | X | S |
| C13-36 ; Predicted metal binding protein | *Z. mobolis* ZM4 (ZMO1965) | 90(92) | 1 | S | S | S |
| TA10; Glutathione S-transferase | *Z. mobolis* ZM4 (ZMO1118) | 99(94) | 1 | X | X | X |
| Others | | | | | | |
| C31-15; TrpR binding protein WrbA | *Z. mobolis* ZM4 (ZMO1335) | 99(96) | 1 | S | S | S |
| TE18; Conserved hypothetical protein | *Z. mobolis* ZM4 (ZMO0695) | 97(97) | 3 | X | X | X |
| TA4 ; Protein unknown function | *Z. mobolis* ZM4 | 99(97) | 1 | X | X | X |

| | | | | | | |
|---|---|---|---|---|---|---|
| DUF162 | (ZMO0021) | | | | | |
| TB10; Conserved hypothetical | *Z. mobolis* ZM4 (ZMO1243) | 98(97) | 2 | X | X | X |
| C12-37; Hypothetical protein | *Z. mobolis* NCIMB 11163 (Za10_0951) | - (84) | 2 | X | S | S |

The values in parentheses represent the identities (%) of the thermotolerant genes compared with the genome of *Z. mobilis* NCIMB 1163

[(a)]According to the data of Agar diffusion assay, "S" represents difference on growth between the thermosensitive mutants compared with the *Z. mobilis* TISTR 548 and "X" represent on difference. Osmotic stress, ethanol stress and oxidative stress is YPD medium supplemented with 12% w/v glucose, 2.5% w/v ethanol and 2 mM $H_2O_2$, respectively, as descript in material and methods.

### 3.3.3 Identification of possible thermotolerant genes

The insertion site of Tn*10* in the genome of each thermosensitive mutant was determined by thermal asymmetric interlaced (TAIL)–PCR and nucleotide sequencing. This determination revealed the genomic sequences flanking Tn*10* inserted in the genome (Table 3.2). The nucleotide sequences were then compared with the genome sequences of the type strain ZM4 [Seo et al., 2005] and NCIMB 11163 [Kouvelis et al., 2009]. Genes disrupted by the insertion of Tn*10* were assumed to be thermotolerant genes if there was no polar effect of Tn*10* insertion on the downstream genes, and thus 42 possible thermotolerant genes were identified by the analysis. Of these, 23 genes were found to be duplicated, and 19 unique genes were identified. Interestingly, one gene encoding transposase is absent from the type strain ZM4 genome. This is not surprising because there is the different number of genes present in the same specie, which allows each strain to exhibit individual characteristics [Welch et al., 2002; Seo et al., 2005; Kouvelis et al., 2009].

### 3.3.4 Effect of other stresses on thermotolerant mutants

In *E. coli*, about 60% of thermotolerant genes have been shown to be oxidative-stress tolerant genes (see Chapter 2). Effects of osmotic, ethanol and oxidative stresses were thus examined on YPD plates. As expected, a part of the thermosensitive mutants also exhibited defective growth on other stresses (Table 3.2). Therefore, it is assumed that there is some correlation between high temperature and other stresses in *Z. mobilis* as reported in a conventional yeast [Auesukaree et al., 2009] and *E. coli* [Gunasekera et al., 2008].

## 3.4 Discussion

Thermotolerant *Z. mobilis* TISTR 548, which was found to be an efficient ethanol producer at high temperature compared to other type strains, was subjected to transposon mutagenesis to obtain thermosensitive mutants at CHT. The analysis of thermosensitive mutants led to identify 17 possible thermotolerant genes. On the basis of the evidence that *Z. mobilis* possesses about 2,000 genes in its genomes [Seo et al., 2005; Kouvelis et al., 2009], the ratio of possible thermotolerant genes to total genomic genes in *Z. mobilis* was about 0.85%, which was nearly equivalent to that in *E. coli* (see Chapter 2). The possible thermotolerant genes were functionally categorized into 8

groups as shown below. Notably, all groups categorized except for anti-oxidative stress were found in *E. coli*, suggesting that a similar molecular mechanism functions in survival at CHT.

(i) Membrane stabilization: squalene hopene cyclase (ZMO1548) encoded by *shc* is involved in the biosynthesis of pentacycline triterpanoid lipids, known as a hopanoids biosynthesis pathway. Hopanoids is the prokaryotic membrane component [Hermans et al., 1991] equivalent to sterols in eukaryotes and is thought to protect bacterial cells by reducing molecular permeability and increasing membrane stability from environmental stresses, such as high temperature, high ethanol concentrations, or high oxygen levels [Ourisson and Rohmer, 1992; Berry et al., 1993]. It has been thought that the acquisition of hopanoids is an evolutionary adaptation for survival in the presence of ethanol in *Z. mobilis* [Carey and Ingram, 1983]. ZMO056 is glucosamine/fructose-6-phosphate aminotransferase (GFAT; EC 2.6.1.16) and catalyzes the formation of glucosamine-6-phosphate using glutamine as an ammonia donor. GFAT is the first and rate-limiting enzyme in the hexosamine biosynthetic pathway (HBP). This nucleotide sugar is essential for the formation of a plethora of glycoconjugates for the peptidoglycan macromolecule in prokaryotes [Badet et al., 1987]. The disruption of GFAT gene is vital in some species, but these mutants can resume growth when glucosamine is added to the media [Smiht et al., 1996; Bulik et al., 2003; Authur et al., 2004]. The other is a membrane-spanning protein TolQ (*ZMO0161*) which is one component for a complex structure forming a biopolymer transporter [Kampfenkel and Braun 1993]. The mutation of this gene suffers delayed outer membrane (OM) invagination and contains large OM blebs at constriction sites and cell poles [Gerding, 2007].

(ii) DNA repair: DNA repair protein RadC encoded by *radC* (*ZMO0663*) functions specifically in recombination repair that is associated with the replication fork and required for growth-medium-dependent repair of DNA strand breaks like *recA* that required for virtually all recombination stimulated by *dnaB107* [Saveson and Lovett, 1999]. During competence, *radC* is specifically co-induced with six flanking genes, *maf*-(*radC*)-*mreBCD*-*minCD*, encoding proteins involved in cell shape determination and septum placement. RadC thus could have a function related to cell envelope metabolism [Attaiech et al., 2008]. Exonuclease VII large subunit XseA encoded by *xseA* (*ZMO0300*) is implicated in the resection of the nicked mismatched strand during bacterial methyl-directed mismatch repair pathway [Harris et al., 1998] that is used to repair base mutations thereby keeping genome mutation frequencies low [Larrea et al., 2008]. Unexpected, all two mutant strains in this group were not sensitive to 2 mM

$H_2O_2$. This may be explained by the fact that *Z. mobilis* as a facultative bacterium must have defense mechanisms against oxidative stress [Seo et al., 2005] and the severe growth was observed under coupled stresses of 2 mM $H_2O_2$ with high temperature (data not shown). Intracellular ROS that causes the DNA damage [Condon, 1987; Zagorski, 2009] increases as temperature increases [Noor et al., 2009]. Cells could maintain their survival ability under such conditions by DNA repair system.

(iii) tRNA modification: tRNA/rRNA methytransferase SpoU encoded by *ZMO1328* is involved in modification of tRNA molecules and may contribute to stabilization of ribosome three-dimensional structure [Decatur and Fournier 2002]. A number of additional modifications in RNAs provide advantages under particular conditions, such as conferring resistance against ribosome-targeting antibiotics [Mann et al., 2001; Toh et al., 2008]. However, the physiological roles of the vast majority of modified nucleosides in rRNA remain unknown [Ero et al., 2008]. Hereby, I propose that tRNA methyltransferase is indispensable for the growth of *Z. mobilis* at CHT.

(iv) Chaperon/protease: metallopeptidase encoded by *ZMO1422* contributes to the cellular process of regulating heat shock response in the wide-range organisms including mammalian and bacteria [Huang et al., 2008].

(v) Translation: ZMO1897 is a subunit of pre-protein translocate involved in protein secretion to the periplasmic space or outer membrane.

(vi) Cell division: ZMO0236 is one of ATPases functioning as a chromosome partitioning–like protein and may be essential for conversion of chromosome dimer to monomer during cell division [Leonard et al., 2005].

(vii) Anti-oxidative stress: xanthine-uracil permease encoded by *ZMO0969* is involved in purine salvage and metabolism [Christiansen et al., 1997]. Xanthine is one of a subset of all bases of DNA damage generated by ROS [Kow, 2002]. Oxidized xanthine by xanthine oxidase was generated by periplasmic superoxide anions [Borsetti et al., 2005], that causes damage to membrane lipids, cellular proteins and DNA molecules. In 42 thermosensitive mutants, 21 xanthine-uracil permease mutants were obtained, which implied that this gene was the hot spot for insertion site for transposon Tn*10* in *Z. mobilis*. *ZMO1965* encodes a predicted metal-binding protein. *ZMO0935* encodes glutathione-S-transferase. This enzyme family is a superfamily of enzymes that play a crucial role in cellular detoxification such as biotransformation of toxic compounds, protection against several stresses and antibacterial drug resistance [La Roche and Leisinger, 1990; Masai et al., 1993; Favaloro et al., 2000; Orser et al., 1993].

(viii) Unknown function: noteworthy, about 25% of the identified genes encode unknown proteins. TrpR-binging protein WrbA (*ZMO1335*), DUF162 (*ZMO0695*),

conserved hypothetical proteins (*ZMO0021*) and (*ZMO1243*), and hypothetical protein (*Za10_0951*). Interestingly, WrbA functions as an accessory element in blocking TrpR-specific transcriptional process [Yang et al., 1993] of which expression increased at the early stage of the stationary phase. It could play a role in preparing the cell for long-term maintenance under stress conditions [Chang, 2002]. WrbA is also reported as the multimeric flavodoxin-like proteins family [Grandori, 1998] and designated as a new type (type IV) of NAD(P)H:quinone oxidoreductases which protect cells against oxidative stress [Patridge and Ferry, 2006] under stationary phase or at high temperature [Soares et al., 2010; Noor et al., 2009].

Surprisingly, the latest protein is hypothetical protein (Za10_0951), which is absent in the genome of the type strain ZM4 and shares a high similarity to transposase (GOX2685) in *Acetobacter pasteurianus* IFO3283 [Asuma et al., 2009]. Transposase is one of DNA-binding enzymes and a member in the polynucleotidyl transferase superfamily that catalyzes 'cut-and-paste' or 'copy-and-paste' reactions, resulting in movement of DNA segments to new sites [Rice and Baker, 2001]. It thus leads to mutations and rearrangements and possibly accelerates biological diversification and consequently evolution [Aziz et al., 2010] by horizontal gene transfer [David et al., 2003]. Therefore, it is speculated that the hypothetical protein Za10_0951 is a transposase that implicates to the cell survivability at CHT.

As described in Chapter 2, 51 thermotolerance genes were discovered in *E. coli* and classified into 8 groups (A-H) [Murata et al., 2011 *inpress*]. Interestingly, of those 6 groups matched with groups of thermotolerant genes in *Z. mobilis*. There is no gene classified into group A of energy metabolism in *Z. mobilis* genome. This may be due to the different of the medium used, LB for *E. coli* and YPD for *Z. mobilis*. The latter medium contains sufficient glucose. Additionaly, genes belong to anti-oxidative stress response genes found in *Z. mobilis* which absent in *E. coli* thermotolerant gene categories [Murata et al., 2011 *inpress*].

Interestingly, there is overlapping of thermotolerant genes with the genes for other stresses (Table 3.2) such as the osmotic stress. Overlapping between the genes for thermotolerance and genes for oxidative stress was found in *E. coli*, but the mechanism is not adequately understood [Gunasekera et al., 2008]. Findings in this study would be useful for generating microbes for high temperature fermentation which expected to the improvement of production of ethanol or other useful materials.

# CHAPTER 4

## Physiological importance of cytochrome *c* peroxidase in ethanologenic thermotolerant *Z. mobilis*


### Abstract

*Z. mobilis* ZmCytC as a peroxidase bearing three heme *c*-binding motifs was investigated with *ΔZmcytC* constructed. The mutant exhibited filamentous shapes and reduction in growth under a shaking condition at a high temperature compared to the parental strain and became hypersensitive to exogenous $H_2O_2$. Under the same condition, the mutation caused increased expression of genes for three other antioxidant enzymes. Peroxidase activity, which was detected in membrane fractions with ubiquinol-1 as a substrate but not with reduced horse heart cytochrome *c*, was almost abolished in *ΔZmcytC*. Peroxidase activity was also detected with NADH as a substrate, which was significantly inhibited by antimycin A. NADH oxidase activity of *ΔZmcytC* was found to be about 80% of that of the parental strain. The results suggest the involvement of ZmCytC in the aerobic respiratory chain via the cytochrome $bc_1$ complex in addition to the previously proposed direct interaction with ubiquinol and its contribution to protection against oxidative stress.


### 4.1 Introduction

*Z. mobilis,* a Gram-negative and facultative anaerobe in alpha-proteobacteria, is known to be an efficient ethanol producer [Swings and De Ley, 1977; Baratti and Bu'lock, 1986; Thanonkeo, 2005]. The organism utilizes the Entner-Doudoroff pathway [Sprenger, 1996] and possesses a relatively simple respiratory chain, consisting of a type-II NADH dehydrogenase, ubiquinone and *bd*-type ubiquinol oxidase [Sootsuwan et al., 2007]. The activity of NADH dehydrogenase is relatively strong and thus the respiratory activity may compete for NAD(P)H with the ethanol production reaction under aerobic conditions [Kalnenieks et al., 2006; Sootsuwan et al., 2007], and the respiratory consumption of NAD(P)H may limit the reduction of acetaldehyde to ethanol, presumably causing accumulation of acetaldehyde to inhibit cell growth [Viikari and Gisler, 1986; Kalnenieks et al., 2008].

Since the ethanol fermentation process is exothermic [Uden, 1981; Ghose, 2004], ethanologenic microorganisms seem to be exposed to heat stress in addition to other stresses including ethanol [Attfield, 1997] and to oxidative stress by endogenous reactive oxygen species (ROS) including hydrogen peroxide ($H_2O_2$) [Auesukaree et al., 2009], which are accumulated more as temperature increases [Noor et al., 2009]. $H_2O_2$ causes damage to membrane lipids, cellular proteins and DNA molecules [Condon, 1987; Zagorski, 2009], and such damage influences cell morphology and cell survival in an additive or synergic fashion [Hartke et al., 1998; Trusca et al., 1998]. Therefore, detoxification mechanisms of oxidative stress may be important not only for survival of these organisms but also for ethanol production. Several genes responsive to oxidative stress in *Z. mobilis* are present in its genome: *ZmcytC* for cytochrome *c* peroxidase, *ZMO1573* for iron-dependent peroxidase, *Zmcat* for catalase, *Zmsod* for superoxide dismutase and *ZmahpC* for alkyl hydroperoxide reductase [Seo et al., 2005].

Cytochrome *c* peroxidase (CCP), which plays an important role in protecting organisms from damage caused by $H_2O_2$, is distributed widely in eukaryotes and prokaryotes. The structural and functional understanding of cytochrome *c* peroxidase has been greatly advanced after the first purification from baker's yeast by Altschul et al. [1940]. Unlike yeast CCP possessing a single *b*-type heme as a co-factor, bacterial CCPs (bCCPs) contain *c*-type heme. Bacterial genome sequencing, however, has revealed that bCCP is present in many but not all Gram-negative bacteria and appears to be absent in Gram-positive bacteria or Archaea. This might be related to the lack of a periplasmic compartment in these organisms [Atack and Kelly, 2007]. Conventional bCCPs are periplasmic enzymes that contain two covalently bound *c*-type hemes, thus called di-heme CCP [Fülöp et al., 1995; Shimizu et al., 2004; Dias et al., 2004; Echalier et al., 2006]. One of the two hemes functions as the peroxidation site where $H_2O_2$ binds. The second heme is the site accepting electrons from a physiological donor such as cytochrome $c_{551}$ in *Pseudomonas aeroginosa* [Hsiao et al., 2007] and cytochrome $c_2$ in *Rhodobacter sphaeroides* [Abresch et al., 2008]. Interestingly, there are bCCPs with three heme-binding motifs in *E. coli* and related endobacteria such as *Gluconobacter oxydans*, *Bacteroides fragilis* and *Z. mobilis*. They bear an N-terminal extension with a heme *c*-binding motif (CXXCH) and a putative methionine ligand [Atack and Kelly, 2007]. The tri-heme CCP in *Actinomyces actinomycetemcomitans* has been demonstrated to be a quinol peroxidase based on evidence that it utilizes reduced ubiquinone-1 as an electron donor for the reduction of $H_2O_2$ to water [Yamada et al., 2007; Takashima et al., 2010].

*Z. mobilis* cytochrome *c* peroxidase (ZmCytC) shares homology with tri-heme CCPs and di-heme CCPs [Atack and Kelly, 2007]. Since *Z. mobilis* possesses the $bc_1$ complex and cytochrome $c_{552}$ without cytochrome *c* oxidase, ZmCytC is speculated to accept an electron(s) from the $bc_1$ complex via cytochrome $c_{552}$ (ZMO0961) to convert hydrogen peroxide to water in the periplasm [Sootsuwan et al., 2007]. However, no direct evidence for this speculated electron transfer process has been provided.

In this study, we constructed a knockout mutant of *ZmcytC* to elucidate its physiological function. Effects of mutation on growth and morphology and on the expression of genes for other antioxidant enzymes were examined. Biochemical analysis was also performed to determine whether ZmCytC catalyzes peroxidase reaction with reduced ubiquinol-1 or NADH as an electron donor in membrane fractions. The NADH-dependent peroxidase activity was inhibited by the addition of antimycin A. On the basis of these results, we discuss the physiological function of ZmCytC and its possible electron donor in *Z. mobilis*.

## 4.2 Materials and methods

### 4.2.1 Materials

Restriction enzymes and T4 DNA ligase were purchased from Takara Shuzo (Kyoto, Japan) and New England Biolabs. A DNA sequencing Kit (ABI PRISM ® Terminator v 3.1 Cycle sequencing Kit) was obtained from Applied Biosystem Japan. Oligonucleotide primers (Table 4.1) were synthesized by Proligo Japan K.K. (Tokyo, Japan). Other chemicals were all of analytical grade and obtained from commercial sources.

**Table 4.1** Primers used in this study

| Primers | Sequence (5' to 3') |
|---|---|
| cytC-5 | ctctgaaagccagaacc |
| cytC-up-kan-3 | cagctccagcctacacagaggttgttgccccata |
| cytC-down-kan-5 | aaggaggatattcatatgctatcgcggccataatc |
| cytC-3 | tcgccatcaaaagcacg |
| Kan-cytC-5 | tatggggcaacaacctctgtgtaggctggagctg |
| Kan-cytC-3 | gattatggccgcgatagcatatgaatatcctcctt |
| cytC-5RT | cttctgtgatcgttgcc |
| cytC-3RT | aaaggtctatggcggtc |
| sod-5RT | cccagacatcgagcgtc |
| sod-3RT | tacccgccctgcccttt |
| cat-5RT | gcccaaccctcctccaa |
| cat-3RT | atgccctgttgtgacag |
| ahpc-5RT | gcaagggcaaaggtcat |
| ahpc-3RT | gttcagcagggtgtatc |
| iron-5RT | ccgaacccctgccccaa |
| iron-3RT | tcttcaccatcccgctc |
| TAIL-840up1 | ccaagacagaaagcacatcc |
| TAIL-840up2 | cgctgtcatatcgccat |

### 4.2.2 Bacterial strains, culture conditions and membrane preparation

Bacterial strains and plasmids used in this study are shown in Table 4.2 *Z. mobilis* TISTR 548 and its mutant, *ΔZmcytC*, were grown in YPD medium consisting of 0.3% yeast extract, 0.5% peptone and 3% glucose [Michel et al., 1985] at 30 °C or 37 °C under a shaking condition at 200 rpm or under a static condition [Taherzadeh et al., 1997]. Cells cultured for 9 h and 24 h (corresponding to optical densities of about 0.8 and 2.5 at 550 nm under the shaking condition and about 1.5 and 3.0 at 550 nm

under the static condition, respectively) were used for some experiments as those in exponential and stationary phases, respectively. To prepare membrane fractions, cells were harvested and suspended in 10 mM potassium phosphate buffer (pH 7.0) and then passed twice through a French press at 16,000 psi, followed by centrifugation at 86,000 *g* for 90 min [Sootsuwan et al., 2007]. The pellet was then homogenized with the same buffer and used as a membrane fraction.

**Table 4.2** Bacterial strains and plasmids used in this study

| Strain or plasmid | Genotype | Reference |
|---|---|---|
| *E. coli* DH5α | $F^-\psi 80\Delta lacZ\Delta M15\Delta$ (*lacZYA-argF*) *U169 recA*1 *endA*1 *hsd*R*17*($r_k^-$, $m_k^+$) *phoA supE44* $\lambda^-$*thi*1 *gyrA96 relA*1 | Novagen |
| *Z. mobilis* TISTR 548 | Wild type | TISTR |
| *Z. mobilis* T548ΔcytC | *Z. mobilis* TISTR 548 Δ*Zmcyt*C | Present work |
| **Plasmid** | | |
| pGEM-T Easy | $Amp^r$ | Promega |
| pDK4 | $Km^r$ | K. A. Datsenko |
| pGEMZMCYTC::Km | pGEM-T Easy carrying a fragment of 3.4-kb; sequence upstream and downstream kb of *Zmcyt*C with insertion of 1.5-kb of kanamycin resistance gene | Present work |
| pZA22 | $tet^r$, $cml^r$ | H. Yanase |
| pZAZMCYTC | pZA22 carrying a 3.5-kb fragment of the full length of *Zmcyt*C gene between the *Bam*HI and *Sal*I of pZA22 | Present work |

### 4.2.3 DNA manipulation

Conventional recombinant DNA techniques were applied [Sambrook and Russell, 2001]. To construct an expression plasmid of *ZmcytC*, we designed primers (Table 4.1) specific to the region about 840 bp upstream from the initiation codon of *ZmcytC* and the region 1,100 bp downstream from its termination codon, on the basis of the genome sequence of *Z. mobilis* strain ZM4 strain [Seo et al., 2005]. Using a primer set of cytC-5 and cytC-3, a 3,462-bp DNA fragment was amplified by PCR with PrimeSTAR (TAKARA BIO INC, Shiga, Japan) and *Z. mobilis* TISTR 548 genomic DNA as a template. The amplified DNA fragment was cloned into pGEM-T Easy. The construct was further confirmed by nucleotide sequencing [Sanger et al., 1977]. The plasmid DNA was digested with *Bam*HI and *Sal*I, and DNA fragment containing *ZmcytC* was ligated into the *Bam*HI-*Sal*I site of pZA22, generating pZAZMCYTC. A *ZmcytC*-disrupted mutant strain was constructed by homologous recombination. The recombinant DNA fragment for the homologous recombination was prepared by fusion PCR [Kuwayama et al., 2002]. The DNA fragments of upstream and downstream sequences of *ZmcytC* were separately amplified by PCR using *Z. mobilis* TISTR 548 genomic DNA as a template and a primer set of cytC-5 and cytC-up-kan-3 and a primer set of cytC-down-kan-5 and cytC-3, respectively. PCR reaction was performed with as follows: heating to 98 °C for 1 min and 30 cycles of 98 °C for 10 s, 52 °C for 10 s and 72 °C for 2 min, followed by a final extension at 72 °C for 5 min. The kanamycin resistance cassette (*kan*) was amplified by PCR using pKD4 plasmid DNA carrying the *kan* gene as a template with a primer set of kan-cytC-5 and kan-cytC-3. PCR products were separated on 0.9% agarose gel. The corresponding DNA bands were excised and purified with Easy trap (TAKARA BIO). The Fusion PCR with the three purified DNA fragments was performed using a PuReTaq™ Ready–To– Go™ PCR beads kit (GE Healthcare, Buckinghamshire, UK) as follows: 94 °C for 1 min and 10 cycles of 94 °C for 10 s, 40 °C for 10 s and 72 °C for 4 min, followed by an extension at 72 °C for 5 min. After the process, 10 pmol primers of cytC-5 and cytC-3 were added to the reaction tube and the fusion was restarted as follows: 94 °C for 1 min and 30 cycles of 94 °C for 10 s, 52 °C for 10 s and 72 °C for 4 min, followed by an extension at 72 °C for 5 min. The fusion PCR product was then purified and ligated into pGEM-T Easy. The resultant clone was verified by nucleotide sequencing. The deletion in the *ZmcytC* ORF of the clone was 1,355 bp encompassing from the 23$^{rd}$ codon to the 452$^{nd}$ codon. The plasmid DNA was isolated and introduced into *Z. mobilis* TISTR 548. The

disrupted mutants were screened on YPD agar plates containing kanamycin (50 μg/ml). The *ZmcytC* disruption was confirmed by using the genomic DNA from the mutant as a template and further proved by digestion with *Pst*I, of which a recognition site is present in the *kan* gene. Furthermore, the specific insertion of the recombinant fragment including the *kan* cassette into the genome was examined by using TAIL-PCR [Lui et al., 1995] followed by the nucleotide sequencing, which determined sequences outward from the 840-bp upstream position from the initiation codon of *ZmcytC* and sequences outward from the 1,100-bp downstream position from the stop codon. Briefly, the mutant genomic DNA used as a template for 1st TAIL-PCR with a specific primer of the upstream TAIL-840up1 or the downstream TAIL-1100down1 of *ZmcytC* and arbitrary primer (AD2 or AD3). The 1st PCR products were used as the template for the 2nd TAIL-PCR with a specific primer of the upstream TAIL-840up2 or the downstream TAIL-1100down2 of *ZmcytC* and arbitrary primer (AD2 or AD3). The 2nd PCR products were used as the template for the 3rd TAIL-PCR with a specific primer of the upstream TAIL-840up3 or the downstream TAIL-1100down3 of *ZmcytC* and arbitrary primer (AD2 or AD3), all of the TAIL-PCR amplification was performed as described previously [Lui et al., 1995]. The 2nd PCR product or 3rd PCR product was subjected to nucleotide sequencing on an ABI PRISM310 (PE Biosystems) with a primer of TAIL-840up3 or TAIL-1100down3

### 4.2.4 Halo assay

Halo assays for sensitivity to $H_2O_2$ of the *ΔZmcytC* and its parental strains and their transformant with pZAZMCYTC or an empty vector were performed as described previously [Lertwattanasakul et al., 2009]. Briefly, 1 ml of culture at mid-exponential phase (density to $OD_{550}$= 1) was mixed with 2 ml of soft YPD agar (0.8% agar) and spread on YPD agar plates. Various volumes of 10% (v/v) $H_2O_2$ were placed on the center of the sterilized discs. This paper disc assay was carried out in triplicate cultures for each strain and the data were treated statistically.

### 4.2.5 Intracellular ROS level

An oxidative-sensitive probe, dichlodihydrofluorescein $H_2$DCFDA, was used to measure the intracellular ROS level [Lertwattanasakul et al., 2009]. Briefly, cells were grown in YPD medium with and without 10 μM $H_2$DCFDA at 30 °C or 37 °C

under a shaking condition at 200 rpm for 24 h. Cells were then harvested and washed 3 times with 10 mM potassium phosphate buffer (pH 6.8). They were then subjected to either observation of cell morphology or measurement of fluorescent intensity after disruption by sonication and removal of cell debris. The fluorescent intensity was measured using a Hitachi 650-10S Fluorescence spectrophotometer (excitation, 504 nm; emission, 524 nm). Emission values were normalized by protein concentration.

### 4.2.6 RT-PCR analysis

Cells were grown in YPD medium for 24 h, and total RNA was immediately extracted using the hot phenol method [Aiba et al., 1981]. To examine the expression of *ZmcytC* (ZMO1136), *Zmcat* (ZMO0918), *Zmsod* (ZMO1060), *ZmahpC* (ZMO1732) and ZMO1573, RT-PCR was performed using an mRNA-selective RT-PCR kit (TAKARA BIO), two specific primers for each gene and 0.1 μg of total RNA as described previously [Tsunedomi et al., 2003]. The primer set used for each gene is shown in Table 4.1. After RT reaction had been performed at 40°C for 15 min, PCR consisting of denaturing at 85 °C for 1 min, 45 °C for 1 min and extension at 72 °C for 2 min was carried out. The PCR products after 20, 25, 30 and 35 cycles for each gene were taken and analyzed by 0.9% agarose gel electrophoresis, followed by staining with ethidium bromide. Intensity of bands of RT-PCR products was quantitatively determined using the UN-SCAN-IT gel$^{TM}$ automated digitizing system (Silk Scientific). Linearity of the amplification was observed up to the 25$^{th}$ or 35$^{th}$ cycle. Under our conditions, the RNA-selective RT-PCR was able to specifically detect mRNA because no band was observed when reverse transcriptase was omitted.

### 4.2.7 Analytical procedures

Ethanol and glucose concentrations in the medium were measured by high performance liquid chromatography (HPLC) (Hitachi Chemical, Japan) using Gelpack GL-C610-S (7.8 x 300 mm). The medium fraction was prepared as a supernatant after a low-speed centrifugation of *Z. mobilis* cultures at different cultivation time points and diluted 10 times before applying to HPLC. Using HPLC with a reflective index detector, chromatography was performed at 60 °C and degassed water was used as a mobile phase at a flow rate of 0.3 ml/min. The concentration was then compared with the standard of known concentrations of ethanol or glucose.

Ubiquinol and NADH oxidase activities were measured spectrophotometrically at 25 °C by following the decrease in absorbance at 275 nm and 340 nm, respectively [Elias *et al.*, 2001]. One unit of ubiquinol-1 and NADH oxidase activities is defined as 1 μmol of ubiquinol-1 or NADH oxidized/min, which was calculated with a millimolar extinction coefficient of 12.25 for ubiquinol and 6.25 for NADH. Peroxidase assay was carried out as described previous [Yamada et al., 2007]. To measure ubiquinol or NADH peroxidase activity, the consumption of ubiquinol or NADH was determined in the assay mixture as for the ubiquinol or NADH oxidase expect for the addition of 1 mM glucose and 50 unit glucose oxidase. The reaction was initiated by the addition of 10 mM $H_2O_2$ to the final concentration of 0.01 mM. The cytochrome *c* peroxidase assay was performed at 25 °C in 100 mM Tris-HCl (pH 7.5) and 30 μM horse heart cytochrome *c* [Yamada et al., 2007]. The reaction was initiated by the addition of 10 mM $H_2O_2$. Oxidation of cytochrome *c* was measured at 550 nm.

### 4.2.8 Database search and computer analysis

Database searching was performed with DDBJ, NCBI, Swiss-Prot and TrEMBL databases. Comparison of primary sequences, hydropathy plot analysis and multiple alignments were conducted by using GENETYX (Software Development, Tokyo, Japan).

### 4.2.9 Statistics

Unless otherwise indicated, mean and SD calculated from data of at least three independent experiments are presented. The variations between the experiments were estimated by SD, and the statistical significance of changes was estimated by two-sample *t*-test assuming equal variances. ***, ** and * mean $p < 0.001$, $< 0.01$ and $< 0.05$, respectively.

## 4.3 Results

### 4.3.1 Structural characteristics of ZmCytC

Comparison of the primary structure of ZmCytC, which is deduced from its nucleotide sequence, with primary structures of other bCCPs was performed (Figure 4.1). The length of the amino acid sequence of ZmCytC corresponds to those of tri-heme *c* peroxidases and about 100 amino acid residues including one heme *c*-binding motif extend at its N-terminus compared to sequences of di-heme CCPs. Hydropathy analysis revealed that there is one possible membrane-spanning segment at the N-terminal (data not shown), which is responsible for association with the inner membrane. Although the N-terminal about 20-amino acid sequence has a character of basic amino acids followed by a hydrophobic sequence, similar to that of a signal sequence, it may be not removed because a similar sequence is present in purified tri-heme CCP in *A. actinomycetemcomitans* [Yamada et al., 2007; Takashima et al., 2010]. This is consistent with the finding that most peroxidase activity in *Z. mobilis* was recovered in membrane fractions (see below). ZmCytC shares 39-43% sequence identity with di-heme CCPs and 44-57% with tri-heme CCPs. Yamada et al. [2007] reported that the tri-heme CCP in *A. actinomycetemcomitans* is ubiquinol peroxidase, which is very similar in primary sequence to ZmCytC (47% identity). These findings strongly suggest that ZmCytC is a member of a tri-heme CCP family. However, since it has been annotated as a cytochrome *c* peroxidase in a database, it is also important to clarify the electron donor for ZmCytC.

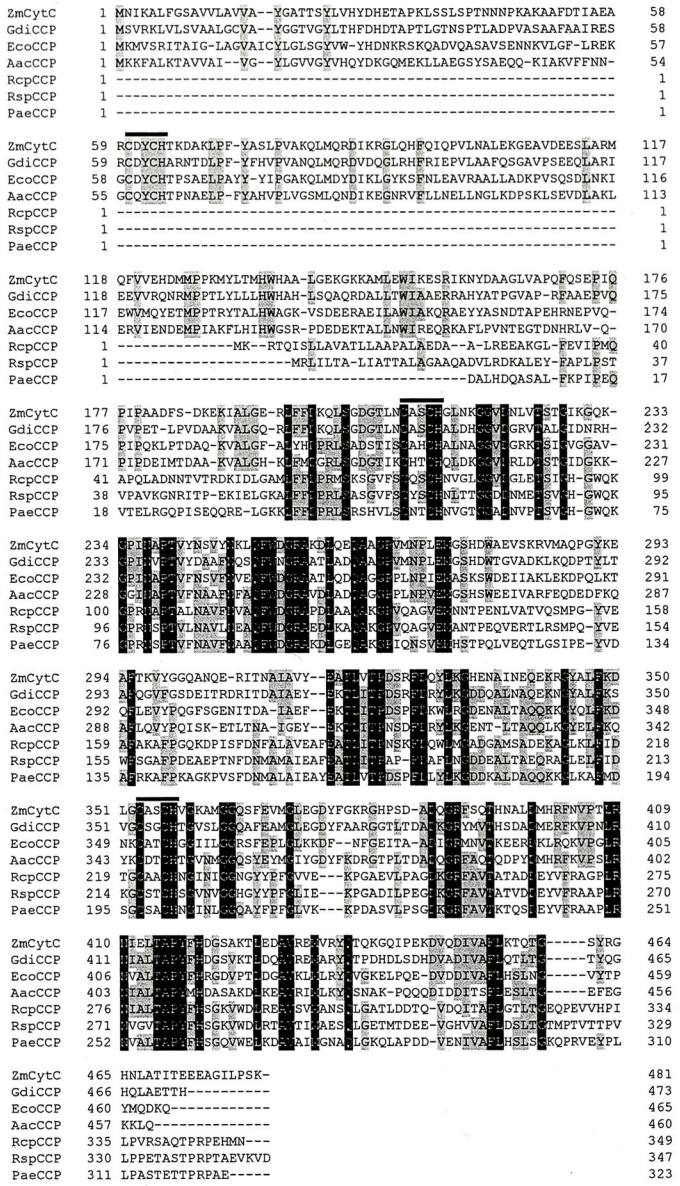

**Figure 4.1** Alignment of deduced amino acid sequences of ZmCytC and other bCCPs. Deduced amino acid sequences of ZmCytC from *Z. mobilis* (NCBI: ZMO1136 ), GdiCCP from *Gluconacetobacter diazotrophicus* PAl5 (NCBI: Gdia_0285 ), EcoCCP from *E. coli* (NCBI: AAC76543.1), AacCCP from *A. actonomycetemcomitans* (DDBJ: AB269691), RcpCCP from *Rh. capsulatus* (DDBJ: RCAP_rcc01723 ), RspCCP from *Rh. sphaeroides* (DDBJ: Rsph17025_1377) and PaeCCP from *P. aeruginosa* bCCP (PDB: 1EB7) were aligned. The parameters were as follows: gap open = 10, gap extension = 0.05, and gap distance = 8. Conserved residues are shadowed. Numbers to the right denote residue positions. Three heme *c*-binding motifs (CXXCH) are indicated by thick upper lines. ZmCytC, GdiCCP, EcoCCP and AacCCP have 3 heme *c*-binding motifs and other three CCPs have 2 heme *c*-binding motifes.

### 4.3.2 Effects of disruption or increased expression of *ZmcytC* on morphology, growth and ethanol production

In order to clarify the physiological function of ZmCytC, we constructed a disrupted mutant of *ZmcytC* by homologous recombination with a *kan* cassette. The specific disruption of the gene was confirmed by TAIL-PCR followed by nucleotide sequencing, which determined the outward sequences both at upstream and downstream junctions between the genome and the DNA fragment inserted for the ZmCytC disruption. Only a single sequence was found at each junction, suggesting that the insertion of the DNA fragment including the *kan* cassette was only one place in its chromosome. *Z. mobilis* TISTR 548 used in this study is thermotolerant and it can grow and produce ethanol even at a relatively high temperature under a shaking condition [Sootsuwan et al., 2007] and under a static condition (unpublished). We thus examined growth and morphology of *ΔZmcytC* at 30 °C and 37 °C because it was expected that cells would be exposed to more oxidative stress as temperature increased [Noor et al., 2009]. When ROS in TISTR 548 was observed by a specific fluorescent probe, dichlodihydrofluorescein $H_2$DCFDA, its level at 37 °C was about 1.7-fold higher than that at 30 °C under shaking conditions, but hardly changed under static conditions (data not shown).

No significant difference in colony morphology between *ΔZmcytC* and its parent was observed on YPD plates or in liquid medium under static conditions. We thus examined the effects of the mutation on growth and morphology in liquid medium under a shaking condition. The growth curves (Figure 4.2A) revealed that the *ZmcytC* mutation caused negative effects on growth at 37 °C and 39 °C, but not at 30 °C. Morphological observation showed that both *ZmcytC* mutation and parental cells became elongated at 37 °C and 39 °C (Figure 4.2B and data not shown) and the mutant cells were a little longer than the parental cells. 4'-6-Diamidino-2-phenylindole (DAPI) staining exhibited a line of nucleoides along with elongated cells at high temperatures (data not shown), suggesting that the elongation is due to the inhibition of cell division. Even at 30 °C, the average cell length of mutant cells was $1.3 \pm 0.2$-fold longer than that of the parental cells.

We further attempted complementation experiments with a plasmid clone of *ZmcytC* (Figure 4.2C and D). However, introduction of an empty vector, pZA22, was found to cause a large reduction of growth at 37 °C though no such effect was found at 30 °C. We have no clear reason for this negative effect of the plasmid. So, due to the

effect, no complementation by pZAZMCYTC was observed in growth at 37 °C (Figure 4.2C). Cells may require more energy to maintain plasmid molecules at a high temperature. With regard to cell morphology, the introduction of pZA22 caused elongation at 30 °C (Figure 4.2D-b and F-b). Both *ΔZmcytC* bearing pZAZMCYTC and the parental strain bearing pZAZMCYTC were found to be shorter in cell size than those bearing an empty vector, pZA22 both at 30 °C and 37 °C, indicating that cell elongation was suppressed by the addition of pZAZMCYTC.

We further examined the effect of the *ZmcytC* mutation on glucose consumption and ethanol production at 30 °C and 39 °C under static and shaking conditions (Figure 4.3). Under static conditions, glucose consumption and ethanol production were faster at 39 °C than at 30 °C. No significant effect of the mutation, however, was observed. Under the shaking condition, glucose consumption was much less at 39 °C than at 30 °C, probably due to repressed growth at a higher temperature (Figure 4.2A). At 30 °C, the *ΔZmcytC* showed 23% and 40% increases in ethanol production at 24 h and 48 h, respectively, compared to those of its parent (Figure 4.3C). These increases may be due to the increase in amount of NADH available for ethanol synthesis since NADH oxidase activity was reduced in the mutant (see below). Taken together, the results suggest that *ZmcytC* is crucial for growth at a high temperature.

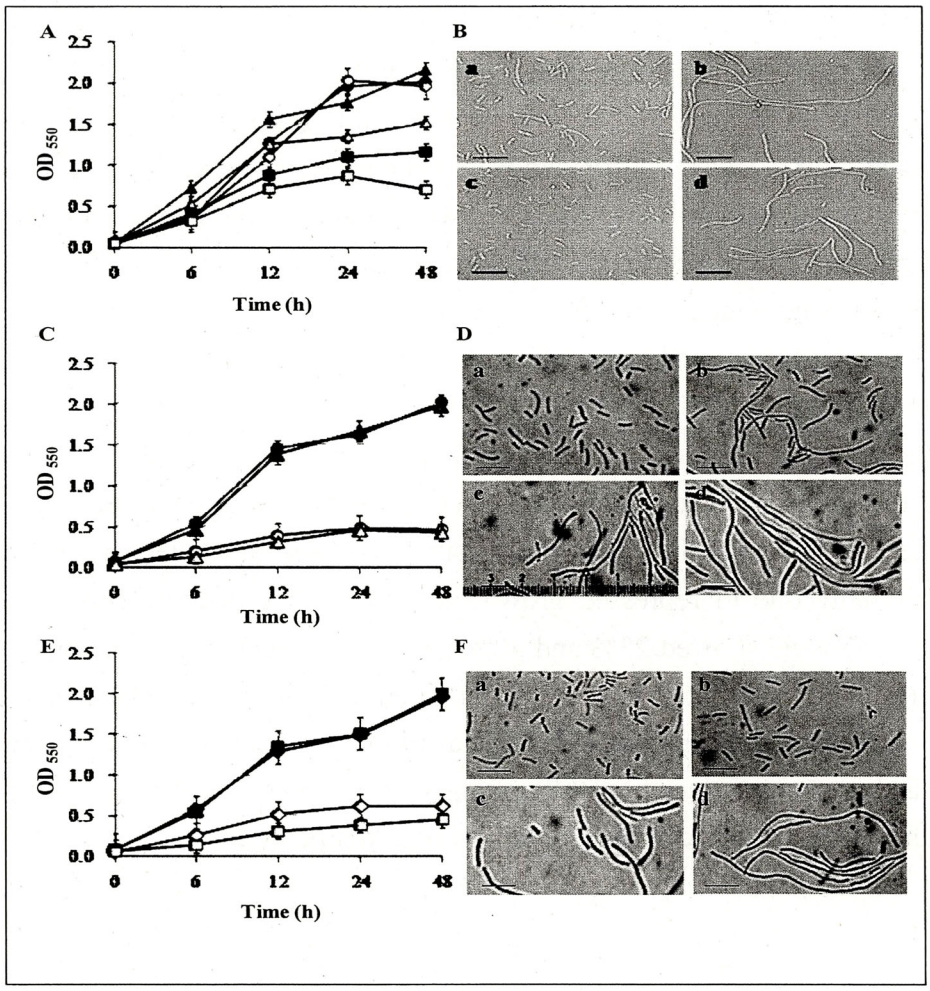


**Figure 4.2** Growth curves and morphology of *Zmcyt*C mutant and parental strains under a shaking condition and effects of *Zmcyt*C clone.

Cells were grown in YPD medium under a shaking condition (200 rpm) at different temperatures and cell morphology at 24 h was observed. **A** Growth curves of *Zmcyt*C mutant (opened symbols) and parental (closed symbols) strains at 30 °C (circles), 37 °C (triangles) and 39 °C (squares). **B** Cells morphology of *Zmcyt*C mutant (**B-a**) and parental strain (**B-c**) at 30 °C and of *Zmcyt*C mutant (**B-b**) and parental strain (**B-d**) at 37 °C in figure **A**. **C** Growth curves of *Zmcyt*C mutant/pZAZMCYTC (circles) and *Zmcyt*C mutant/empty vector, pZA22 (triangles) at 30 °C (closed symbols) and 37 °C (open symbols). **D** Cells morphology of *ZmcytC* mutant/pZAZMCYTC at 30 °C (**D-a**) and 37 °C (**D-c**) and *ZmcytC* mutant /pZA22 at 30 °C (**D-b**) and 37 °C (**D-d**) in figure **C** **E** Growth curves of the parental strain/pZAZMCYTC (diamonds) and the parental strain/pZA22 (squares) at 30 °C (closed symbols) and 37 °C (open symbols). **F** Cells morphology of the parental strain /pZAZMCYTC at 30 °C (**F-a**) and 37 °C (**F-c**) and the parental strain /pZA22 at 30 °C (**F-b**) and 37 °C (**F-d**) in figure **E**. Photos shown in **B**, **D** and **F** are also representatives. Scale bar, 10 μm.

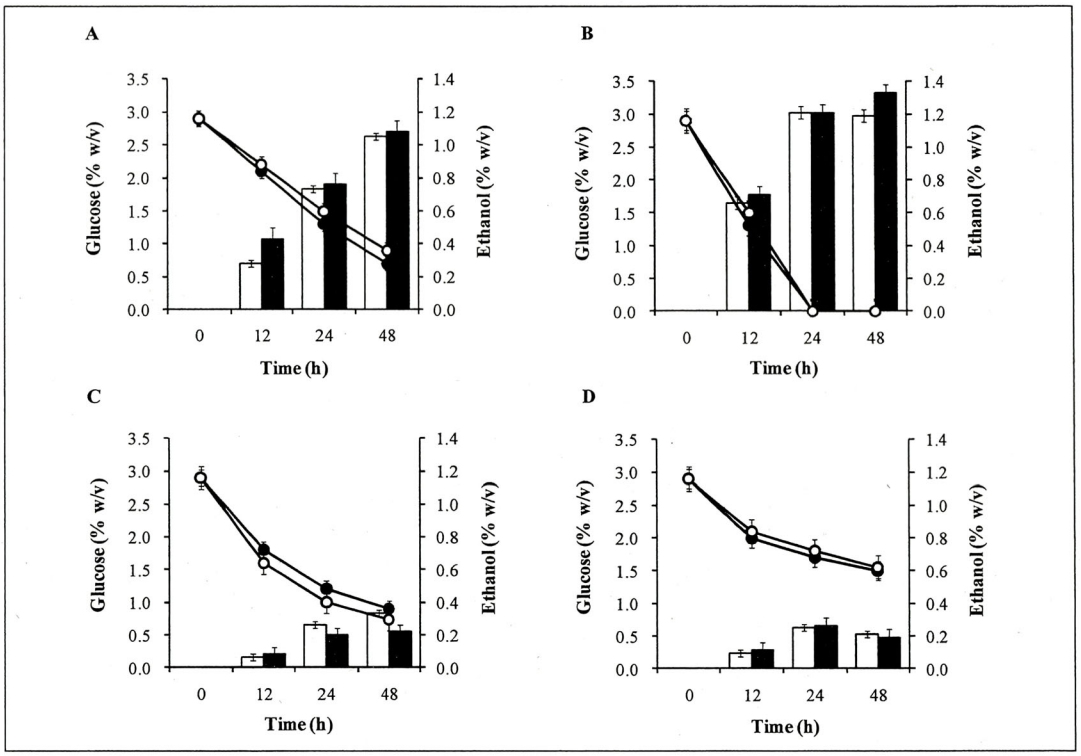


**Figure 4.3** Effect of disruption of *ZmcytC* on glucose utilization and ethanol production. Cells were grown in 30 ml of YPD medium at 30 °C (**A**) or 39 °C (**B**) under static conditions and 30 °C (**C**) or 39 °C (**D**) under a shaking condition (200 rpm). Glucose and ethanol concentrations were determined at the time indicated. Open and closed circles represent glucose concentration in the medium of the *Zmcyt*C mutant and parental strains, respectively. Open and closed columns represent ethanol concentration on the *Zmcyt*C mutant and parental strains, respectively.

### 4.3.3 Sensitivity to $H_2O_2$ of a disrupted mutant of *ZmcytC*

Since *ZmcytC* was expected to encode cytochrome *c* peroxidase, the sensitivity to $H_2O_2$ of *ΔZmcytC* was examined on plates containing filter discs that absorbed different volumes of 10% $H_2O_2$ (Figure 4.4A and B). *ΔZmcytC* showed halos larger than those of the parental strain, suggesting that the mutation caused cells to be hypersensitive to $H_2O_2$. The effect of *ZmcytC* on sensitivity to $H_2O_2$ was further examined by introduction of pZAZMCYTC into *ΔZmcytC* (Figure 4.4C) and its parent (Figure 4.4D). In the case of *ΔZmcytC*, the introduction of the plasmid clone greatly reduced the halo size compared to the control strain harboring pZA22. Similar but slightly smaller effect was observed in experiments with the parental strain. These

results suggest that ZmCytC contributes to the protection against oxidative stress. Notably, comparison of halo sizes at the same concentration of $H_2O_2$ in Figure 4.4B, C and D revealed that introduction of even an empty vector caused hypersensitivity to $H_2O_2$. Maintenance of the plasmid might be a burden to cells.

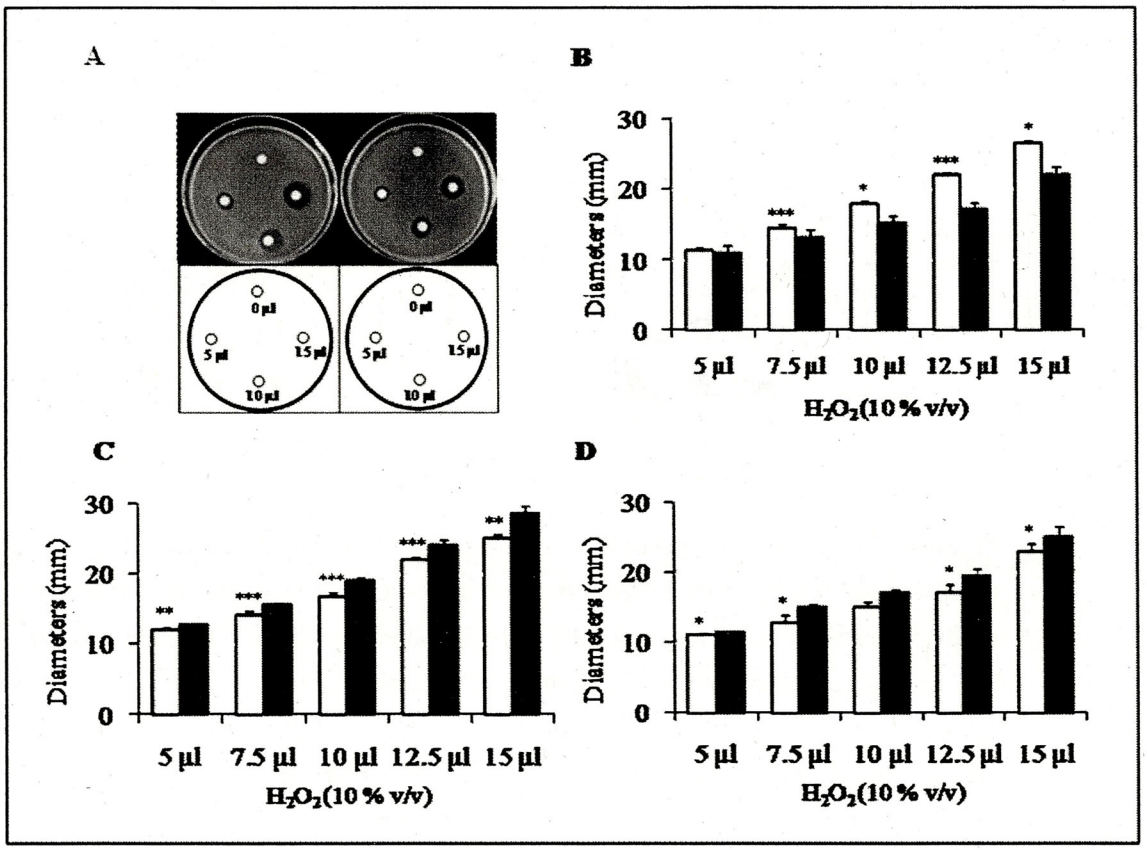


**Figure 4.4** Effect of disruption or increased expression of *Zmcyt*C on sensitivity to exogenous $H_2O_2$.

Disc diffusion assay was performed on YPD plates at 30 °C as described in Experimental Procedures. Typical examples are shown in **A**. Halo diameters determined were shown in **B** for the *Zmcyt*C mutant (open columns) and parental (closed columns) strains, in **C** for *Zmcyt*C mutant/pZAZMCYTC (open columns) and *Zmcyt*C mutant/pZA22 (closed columns) and in **D** for the parental strain/pZAZMCYTC (open columns) and the parental strain/ pZA22 (closed columns). Reported values are the mean (±SD) of three independent experiments. *p* values were calculated by two-sample *t*-test assuming equal variances.

### 4.3.4 Expression of genes presumably related to oxidative stress response and effect of a disrupted mutation of *ZmcytC* on their expression

On the basis of the function of ZmCytC in protection of oxidative stress as described above, it was assumed that the *ZmcytC* disruption affected the expression of genes related to oxidative stress response, *Zmcat*, *Zmsod*, *Zmahp*C and *ZMO1573*. The assumption was examined by RT-PCR with total RNA prepared from cells that were grown in YPD at 30 °C and 37 °C under static or shaking conditions (Figure 4.5). The band intensity in each PCR cycle corresponded to the level of mRNA examined. The intensity of RT-PCR products of *ZmcytC* in the parental strain was stronger at 37 °C than that of 30 °C under both static and shaking conditions and was stronger under the shaking condition than under the static condition at both temperatures. There was no band in *ΔZmcytC*, confirming disruption of the *ZmcytC* gene. The intensity of products of *ZMO1573* was also stronger at a higher temperature under the shaking condition. Therefore, *ZmcytC* and *ZMO1573* are thought to be up-regulated at a higher temperature, a condition in which oxidative stress is more accumulated. On the other hand, the results suggest that *Zmcat* is down-regulated at a high temperature.

Next, the effect of the *ZmcytC* disruption on expression of *Zmsod, Zmcat, Zmahp*C and *ZMO1573* was examined by comparison of the intensities of RT-PCR products from the mutant and the parent. A significant difference in the intensity was observed in *ZmahpC* and *ZMO1573* under the shaking condition at 37 °C and in *Zmsod* and *ZmahpC* under the static condition at 37 °C. These results suggest that *Zmsod, ZmahpC* and *ZMO1573* are complementarily expressed to the *ZmcytC* mutation. ZmCytC may thus be crucial for the protection of *Z. mobilis* from oxidative stress, especially at a high temperature.

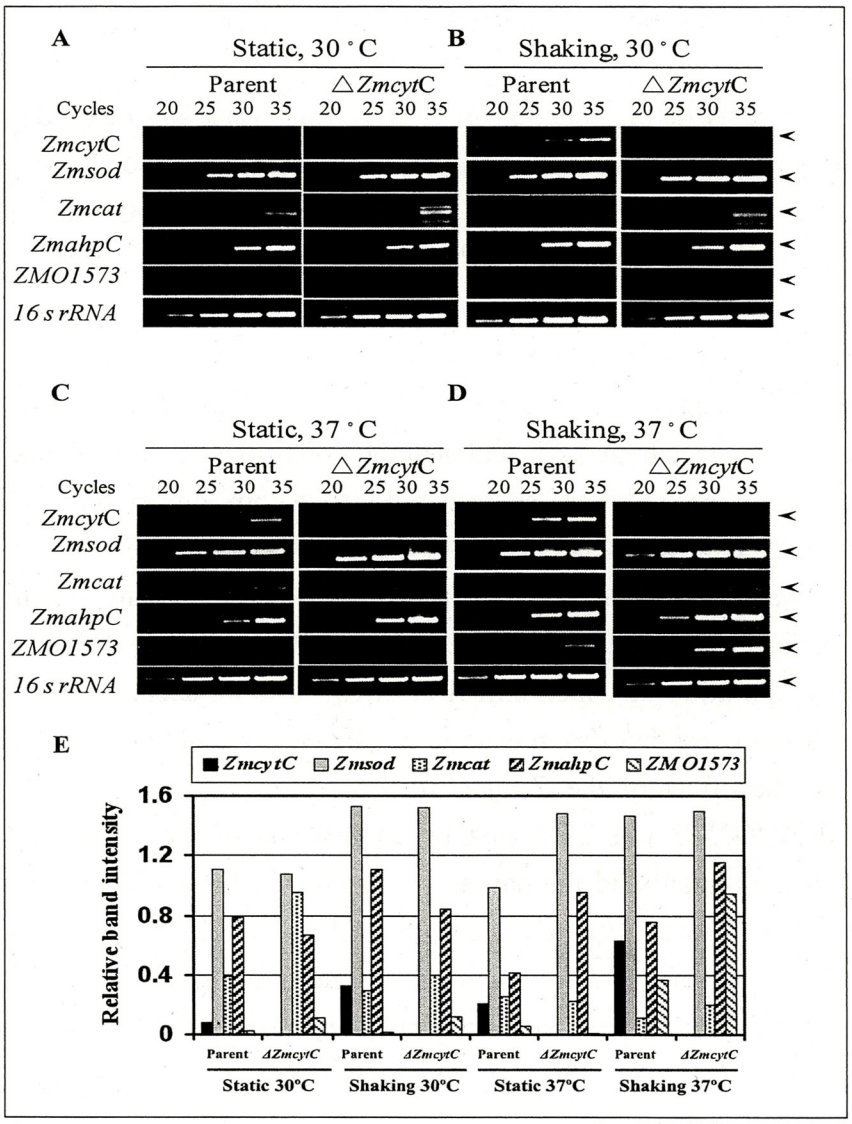


**Figure 4.5** Expression of *ZmcytC*, *Zmsod*, *Zmcat*, *ZmahpC* and *ZMO1573* under static and shaking conditions at 30 °C or 37 °C. *ΔZmcyt*C and the parental cells were grown in YPD medium at 30 °C (**A**) or 37 °C (**B**) under a static and shaking condition (200 rpm) at 30 °C (**C**) or 37 °C (**D**) for 24 h, from which total RNA was prepared. RT-PCR was then carried out with 0.1 μg of total RNA except that 0.001 μg of total RNA was used for 16S rRNA, and PCR products from 20, 25, 30 and 35 cycles were analyzed by agarose gel electrophoresis. **E** Relative band intensity of bands in **A-D** was determined as described in Experimental Procedures.

### 4.3.5 Peroxidase activity of ZmCytC and its possible electron donor

Peroxidase activities of the *ΔZmcytC* and parental strain in membrane fractions were compared (Table 4.3). Ubiquinol-1 peroxidase activity was detected in the parental cells, but only a trace of amount of ubiquinol-1 peroxidase activity was detected in the mutant cells. NADH peroxidase activity in the mutant was also significantly lower than that in the parental cells. These results suggest that ZmCytC functions as a peroxidase in the inner membrane and receives electrons from the respiratory chain.

*Z. mobilis* possesses a simple respiratory chain consisting of dehydrogenase, ubiquinone, and ubiquinol oxidase [Sootsuwan et al., 2007]. The organism, however, retains the cytochrome $bc_1$ complex and possibly its electron acceptor as a cytochrome *c*, though there is no cytochrome *c* oxidase in its genome. We thus examined whether ZmCytC accepts electrons via the cytochrome $bc_1$ complex, by using antimycin A as an inhibitor of cytochrome $bc_1$ complex. The addition of antimycin A greatly reduced NADH peroxidase activity in the parental strain. The remaining activity was nearly the same as the level of NADH peroxidase activity in *ΔZmcytC*. On the other hand, the activity of ubiquinol-1 peroxidase was hardly inhibited by antimycin A, and it is therefore likely that the cytochrome $bc_1$ complex functions as the electron donor for ZmCytC.

**Table 4.3** Peroxidase and oxidase activities in membrane fractions from the parental and *ΔZmcytC* cells

| Strains | Activities (U/mg protein)[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | Cytochrome *c* peroxidase[b] | NADH Oxidase | NADH Peroxidase | $UQ_1H_2$ Oxidase | $UQ_1H_2$ Peroxidase | Antimycin A[c] | |
| | | | | | | NADH Peroxidase | $UQ_1H_2$ Peroxidase |
| **Parent** | ND | 2.04 + 0.13 | 1.30 + 0.20 | 13.62 + 2.08 | 7.91 + 1.10 | 0.37 + 0.01 | 6.90 + 1.33 |
| ***ΔZmcytC*** | ND | 1.60 + 0.39 | 0.44 + 0.05 | 6.80 + 0.50 | 0.35 + 0.00 | - | - |

[a] Cells were grown in YPD medium for 24 h under shaking condition at 30 °C. Preparation of membrane fractions and measurement of enzyme activities were performed as described in Experimental Procedures. Reported values are the mean (±SD) of three independent experiments.

[b] Reduced horse heart cytochrome *c* was used. ND, not detectable,

[c] Activities in the presence of 50 μM antimycin A.

## 4.4 Discussion

ZmCytC in *Z. mobilis* has been annotated as cytochrome *c* peroxidase [Seo et al., 2005], though it seems to possess three heme *c*-binding motifs similar to ubiquinol peroxidase in *A. actinomycetemcomitans* [Yamada et al., 2007; Takashima et al., 2010]. In order to determine the physiological function of ZmCytC and its electron donor, *ZmcytC*-disrupted mutant was constructed and examined. Our findings suggest a unique strategy of the organism to protect cells against $H_2O_2$ in association with respiratory chain.

CCP and ubiquinol peroxidase are thought to contribute to protection against exogenous oxidative stress. The *ZmcytC* mutant was shown to exhibite more sensitivity than the parental strain to exogenous $H_2O_2$ (Figure 4.4) and reduced growth at a high temperature under the shaking condition (Figure 4.2). Since such a growth defect was obvious under the shaking condition but not under the static condition, the defective phenotype may be due to the critical level of endogenously generated $H_2O_2$, which was suggested to be accumulated more at a high temperature. The *ZmcytC* mutant and parental strains became filamentous under the shaking condition at high temperatures. This significant morphology may be due to inhibition of cell division as observed when DNA damage is accumulated. $H_2O_2$ oxidizes solvent-exposed iron, resulting in the formation of hydroxyl radicals which severely damage DNA molecules. In such a situation, cells stop cell dividing and become filamentous [Zagorski, 2009]. The *ZmcytC* mutation caused an increase in the expression of genes for antioxidant enzymes, *Zmsod*, *ZmahpC* and *ZMO1573*, especially at a high temperature. Such a response, however, did not fully suppress the *ZmcytC* mutation under the shaking condition at a high temperature. These finding and the up-regulation of *ZmcytC* at a high temperature suggest that ZmCytC as a $H_2O_2$-degrading enzyme is crucial for survival of *Z. mobilis* at a high temperature.

Structural comparison with other CCPs including the extensively analyzed ubiquinol peroxidase of *A. actinomycetemcomitans* [Yamada et al., 2007; Takashima et al., 2010] suggests that *Z. mobilis* ZmCytC is a membrane-bound peroxidase with three heme *c*-binding motifs. ZmCytC-dependent peroxidase activity was detected in membrane fractions when ubiquinol or NADH, but not reduced horse heart cytochrome

*c*, was used as an electron donor. Surprisingly, NADH peroxidase, but not ubiquinol peroxidase was inhibited by antimycin A, which is known to be an inhibitor for the cytochrome $bc_1$ complex (Table 4.3), suggesting that the $bc_1$ complex mediates the electron transfer from NADH to ZmCytC. Cytochrome $c_{552}$ bearing a putative signal sequence is expected to be an additional mediator [Sootwan et al., 2007]. If this is the case, electrons from the cytochrome $bc_1$ complex via cytochrome $c_{552}$ are fed by ZmCytC to $H_2O_2$ in the periplasm. Considering the report that purified tri-heme CCP in *A. actinomycetemcomitans* catalyzes the peroxidation reaction with ubiquinol as a substrate, ZmCytC may accept electrons from ubiquinol in addition to cytochrome *c*. In the former case, the electron transfer occurs as the order of type II NADH dehydrogenase-ubiquinol-ZmCytC. In the latter case, electrons are transferred as type II NADH dehydrogenase-ubiquinol-cytochrome $bc_1$ complex-cytochrome $c_{552}$-ZmCytC. Since there is no cytochrome *c* oxidase in *Z. mobilis* [Sootsuwan et al., 2007], it is thought that ZmCytC plays an important role in the formation of membrane potential at the cytochrome $bc_1$ complex site. Further study is required for this speculation.

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# Summary

## Molecular mechanism for coping with critical heat and oxidative stresses in thermotolerant *Zymomonas mobilis*

High-temperature fermentation technology with thermotolerant mesophiles is expected to become one of the economical next-generation fermentation technologies, reduces cooling cost, contamination risk and operation cost in addition to advantages specific for ethanol production including higher saccharification and fermentation rates and continuous ethanol removal. For survival at high temperature, thermotolerant mesophiles are expected to possess a mechanism different from non-thermotolerant mesophiles. I thus focused on molecular mechanisms supporting survival at a critical high temperature (CHT) in *Escherichia coli* and thermotolerant *Zymomonas mobilis* that can be alive at 47°C and 39°C, respectively. Additionally, one of strategies for dealing with oxidative stress in *Z. mobilis* was investigated since oxidative stress is increased as temperature is elevated.

*Escherichia coli* genome-wide screening with a single-gene knockout library provided a list of genes indispensable for growth at 47°C, called thermotolerant genes. Genes for which expression was affected by exposure to CHT were identified by DNA chip analysis. Unexpectedly, the former contents did not overlap with the latter except for *dnaJ* and *dnaK*, indicating that a specific set of non-heat shock genes is required for the organism to survive under such a severe condition. More than half of the mutants of the thermotolerant genes were found to be sensitive to $H_2O_2$ at 30°C, suggesting that the mechanism of thermotolerance partially overlaps with that of oxidative stress resistance. Their encoded enzymes or proteins are related to outer membrane organization, DNA double-strand break repair, tRNA modification, protein quality control, translation control or cell division. DNA chip analyses of essential genes suggest that many of the genes encoding ribosomal proteins are down-regulated at CHT. Bioinformatics analysis and comparison with the genomic information of other microbes suggest that *E. coli* possesses several systems for survival at CHT. This analysis allows us to speculate that a lipopolysaccharide biosynthesis system for outer membrane organization and a

sulfur-relay system for tRNA modification have been acquired by horizontal gene transfer.

An ethanologenic thermotolerant *Z. mobilis* TISTR 548 strain that was chosen as a thermotolerant strain was subjected to transposon mutagenesis via transconjugation with a mobilizable plasmid harboring the transposable element (Tn*10*) and growth experiment at 39°C, CHT for the strain. Among about 4,000 of the transconjugants obtained, 42 mutants that were found to be dramatically defective in growth at the CHT, which were selected as thermosensitive mutant strains. The insertion site of Tn*10* within the genome was then determined by thermal asymmetric interlaced-(TAIL) PCR followed by DNA sequencing. As a result, 17 genes related to the thermotolerance have been identified. Some of these were related to membrane biosynthesis and lipid metabolism, recombination for DNA repair and replication, tRNA modification, transportation system, which may have a direct or in direct relation to the thermotolerance mechanism. Interestingly, the results also revealed a partial overlapping between genes required for the thernotolerance and those for tolerance to other stresses. Our findings provide molecular mechanisms underlying a survival of *Z. mobilis* at CHT which may advantages in production of ethanol and other useful materials at high temperature.

*Z. mobilis* ZmCytC as a peroxidase bearing three heme *c*-binding motifs was investigated with *ΔZmcytC* constructed. The mutant exhibited filamentous shapes and reduction in growth under a shaking condition at a high temperature compared to the parental strain and became hypersensitive to exogenous $H_2O_2$. Under the same condition, the mutation caused increased expression of genes for three other antioxidant enzymes. Peroxidase activity, which was detected in membrane fractions with ubiquinol-1 as a substrate but not with reduced horse heart cytochrome *c*, was almost abolished in *ΔZmcytC*. Peroxidase activity was also detected with NADH as a substrate, which was significantly inhibited by antimycin A. NADH oxidase activity of *ΔZmcytC* was found to be about 80% of that of the parental strain. The results suggest the involvement of ZmCytC in the aerobic respiratory chain via the cytochrome $bc_1$ complex in addition to the previously proposed direct interaction with ubiquinol and its contribution to protection against oxidative stress.

# Summary

# (Japanese)

題目：耐熱性 *Zymomonas mobilis* における限界熱ストレスと酸化ストレスに対する対処分子機構 (Molecular mechanism for coping with critical heat and oxidative stresses in thermotolerant *Zymomonas mobilis*)


耐熱性中温菌を用いた高温発酵技術は、高い糖化性や発酵率と連続的エタノール除去を含むエタノール生産に関する利点に加えて冷却コスト削減・雑菌混入抑制・操作コスト削減を可能にする、経済的な次世代発酵技術として注目されている。耐熱性中温菌は、高温下での生存のために非耐熱性中温菌とは異なった機構をもつことが予想される。本研究では47℃で生育可能な *Escherichia coli* と39℃で生育可能な *Zymomonas mobilis* おいて、生育限界温度（CHT）での生存分子機構に焦点を当てた。さらに、温度上昇に伴って酸化ストレスが増加することから、酸化ストレスに対処するための分子機構についても検討した。

*E. coli* のゲノムワイドスクリーニングによって47 ℃での生育に不可欠な遺伝子（耐熱性遺伝子）群を同定した。CHT ショックによって発現変動する遺伝子群を同定した。予想に反して、前者は *dnaJ* と *dnaK* を除いて後者と重複しておらず、CHT では特殊な非熱ショック応答遺伝子セットが要求されることが示された。耐熱性遺伝子変異株の半分以上が30℃で過酸化水素に感受性であることが判明し、耐熱性機構は部分的に酸化ストレス耐性機構と重複していることが示唆された。耐熱性遺伝子は、外膜の形成、DNA 二本鎖切断修復、tRNA 修飾、タンパク質品質管理、転写調節、細胞分裂に関係していた。必須遺伝子の内、多くのリボソームタンパク質遺伝子が CHT で発現抑制されることが示された。生物情報学的解析とゲノム情報比較によって、*E. coli* が生育限界温度での生存のためにいくつかのシステムをもつことが示唆された。特に、リポ多糖の生合成系と tRNA 修飾のための硫黄リレー系が水平伝播によって獲得されてきたと推測された。

エタノール生産性の耐熱性 *Z. mobilis* TISTR 548 株について、トランスポゾン（Tn*10*）変異処理によって42個の CHT 感受性変異株を取得した。ゲノムへの Tn*10* の挿入箇所は、TAIL PCR と DNA シークエンシングによって決定した。そ

の結果、17個の耐熱性遺伝子を同定した。これらの遺伝子は、膜の生合成や脂質代謝、DNA組換え修復と複製、tRNA修飾、輸送系に関与することが分かった。また本研究によって、耐熱性遺伝子群と他のストレス耐性遺伝子群の間で部分的に重複することが示唆された。本研究で明らかになったCHTでの生存分子機構はエタノールや有用物質の高温発酵に活かされると期待される。

*Z. mobilis* のペルオキシダーゼ（ZmCytC）は3つのヘムc結合モチーフをもち、酸化ストレスに対処していると予想される。*ZmcytC* 欠損変異株は、高温撹拌条件下において親株と比較して繊維状形態が顕著となり生育の減少を示し、過酸化水素に対してより強い感受性を示した。*ZmcytC* 欠損変異は、他の3つの抗酸化酵素遺伝子の発現上昇を引き起こした。ペルオキシダーゼ活性はユビキノール-1やNADHを基質として膜画分で検出できた。*ZmcytC* 欠損株ではユビキノール-1-ペルオキシダーゼ活性がほとんど見られなかった。NADH-ペルオキシダーゼ活性はアンチマイシンAによって強く阻害されたが、*ZmcytC* 欠損株ではNADHオキシダーゼ活性は親株の80%程度であった。この結果は以前に提唱されたユビキノールとの直接的相互関係と酸化ストレスに対する防御への貢献に加えて、チトクロム $bc_1$ 複合体を介した好気的呼吸鎖におけるZmCytCの関与を示唆する。

# List of publications

1. **Kannikar Charoensuk**, Akira Irie, Noppon Lertwattanasakul, Kaewta Sootsuwan, Pornthap Thanonkeo and Mamoru Yamada:
**Physiological importance of cytochrome *c* peroxidase in ethanologenic thermotolerant *Zymomonas mobilis*. Journol of Molecular Microbiology and Biotechnology Vol. 20 (2011), No. 2 pp. 70-82 (Chapter 4).**

**2.** Masayuki Murata, Hiroko Fujimoto, Kaori Nishimura, **Kannikar Charoensuk**, Hiroshi Nagamitsu, Satish Raina, Tomoyuki Kosaka, Taku Oshima, Naotake Ogasawara, Mamoru Yamada: **Molecular strategy for survival at a critical high temperature in *Eschierichia coli* Journol of PLoS ONE, in press (Chapter 2)**

**3.** 題目：耐熱性エタノール生産細菌を用いたバイオエタノール生産技術
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