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

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INTRODUCTION

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

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

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

### MATERIALS AND METHODS

#### Bacterial Strains, Media, and Cultivation Conditions

The bacterial strains used in this study are listed in Table 1. To grow Z. mobilis, a preculture was prepared in 2 mL of YPD medium (0.3% yeast extract, 0.5% peptone, and 3% glucose) and incubated overnight at 30°C. The overnight culture was subsequently inoculated into fresh YPD medium at an OD$_{550}$ of 0.05. Cultivation was performed under non-shaking (static) conditions.

#### Examination of the Effects of Various Materials on Cell Growth

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

### Table 1 | List of Zymomonas mobilis strains used in this study.

| Strain | Genotype | Reference or source |
|--------|----------|---------------------|
| TISTR 548 | TISTR 548 (ZZ6_0707:Tn10) | TISTR collections |
| TC01 | TISTR 548 (ZZ6_1376:Tn10) | Charoensuk et al., 2017 |
| TC02 | TISTR 548 (ZZ6_1146:Tn10) | Charoensuk et al., 2017 |
| C12-36 | TISTR 548 (ZZ6_1551:Tn10) | Charoensuk et al., 2017 |
| C11-44 | TISTR 548 (ZZ6_1046:Tn10) | Charoensuk et al., 2017 |
| C13-36 | TISTR 548 (ZZ6_1210:Tn10) | Charoensuk et al., 2017 |
| TC04 | TISTR 548 (ZZ6_0923:Tn10) | Charoensuk et al., 2017 |
| 1-2 | TISTR 548 (ZZ6_1043:Tn10) | Charoensuk et al., 2017 |
| 3-24 | TISTR 548 (ZZ6_0929:Tn10) | Charoensuk et al., 2017 |
| TC14 | TISTR 548 (ZZ6_0158:Tn10) | Charoensuk et al., 2017 |
| C31-23 | TISTR 548 (ZZ6_1254:Tn10) | Charoensuk et al., 2017 |
| C15 | TISTR 548 (ZZ6_1477:Tn10) | Charoensuk et al., 2017 |
| F32 | TISTR 548 (ZZ6_0616:Tn10) | Charoensuk et al., 2017 |
| C12-43 | TISTR 548 (ZZ6_0934:Tn10) | Charoensuk et al., 2017 |
| C10 | TISTR 548 (ZZ6_0681:Tn10) | Charoensuk et al., 2017 |
| C12-44 | TISTR 548 (ZZ6_0023:Tn10) | Charoensuk et al., 2017 |
| C21-17 | TISTR 548 (ZZ6_1659:Tn10) | Charoensuk et al., 2017 |
| TC05 | TISTR 548 (ZZ6_0980:Tn10) | Charoensuk et al., 2017 |
| TC12 | TISTR 548 (ZZ6_0702:Tn10) | Charoensuk et al., 2017 |
| TE19 | TISTR 548 (ZZ6_0979:Tn10) | Charoensuk et al., 2017 |
| C31-15 | TISTR 548 (ZZ6_0019:Tn10) | Charoensuk et al., 2017 |
| TC11 | TISTR 548 (ZZ6_0840:Tn10) | Charoensuk et al., 2017 |
| C12-37 | TISTR 548 (ZZ6_0962:Tn10) | Charoensuk et al., 2017 |
| TC09 | TISTR 548 (ZZ6_0541:Tn10) | Charoensuk et al., 2017 |
| TC13 | TISTR 548 (ZZ6_0861:Tn10) | Charoensuk et al., 2017 |
| 1-10 | TISTR 548 (ZZ6_1289:Tn10) | Charoensuk et al., 2017 |
Cell Morphology

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

Intracellular Reactive Oxygen Species Level

*Zymomonas mobilis* TISTR 548 cells were grown on YPD medium at 39°C. At 12 h, 5 µM H2DCFDA was added to the first culture, and further cultivation was performed at 39°C for 30 min. Then, cells were harvested by low-speed centrifugation and washed once with phosphate-buffered saline [130 mM NaCl, 10.8 mM Na2HPO4, 4.2 mM NaH2PO4 (pH 7.2)]. The washed cells were disrupted by sonication for 30 min using an ultrasonic cell disruptor (Bioruptor; Cosmo Bio, Tokyo, Japan) and subjected to low-speed centrifugation. Supernatant fluorescence was measured using a microplate reader (POWERSCAN® HT; BioTek Instruments, Inc., Winooski, VT, United States). Protein concentration was determined using the Lowry method (Dulley and Grieve, 1975). The result obtained for intracellular reactive oxygen species (ROS) levels is expressed as fluorescence intensity per protein concentration, and the ratio of the number of cells grown in the presence of a metal ion to that of cells grown in its absence was estimated and expressed as percentage.

**FIGURE 1** | Effects of various metal ions on the two-step cultivation of *Zymomonas mobilis* TISTR 548. Cells were cultivated in YPD medium at 38°C with different concentrations of NiCl2 (A), ZnCl2 (C), FeCl3 (E), AlCl3 (G), MnCl2 (I), or CoCl2 (K) and at 39°C with NiCl2 (B), ZnCl2 (D), FeCl3 (F), AlCl3 (H), MnCl2 (J), or CoCl2 (L) under a static condition. These symbols indicate the means of three replicates, and error bars indicate standard deviations: closed circle, control (0 mM); open circle, 0.01 mM; open diamond, 0.1 mM; and open square, 1.0 mM. Dotted and solid lines indicate the OD values of the first and second stages of cultivation, respectively.
Ethanol Concentration
Ethanol concentration was analyzed using a gas chromatograph (GC-2014, Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and Gaskupack 54-packed glass column (60/80 mesh; GL Science, Tokyo, Japan); nitrogen was used as a carrier gas (flow rate, 35 mL/min). Operating temperatures were as follows: injection temperature, 200°C; column temperature, 180°C; and detector temperature, 200°C.

RESULTS
Effects of Metal Ions on *Z. mobilis* TISTR 548 Growth at CHT
To explore the metal ions that enhance *Z. mobilis* TISTR 548 growth at putative CHT, the growth levels with and without the addition of Ni^{2+}, Zn^{2+}, Fe^{3+}, Al^{3+}, Mn^{2+}, Co^{2+}, Mg^{2+}, and K^{+} were compared. The effect of the addition of metal ions was evaluated with two-step cultivation, wherein only viable and culturable cells grow, whereas dead or viable but non-culturable cells do not grow in fresh medium at the second cultivation (Kosaka et al., 2019). At 38°C and 39°C, the growth level under the conditions of 0.01 mM NiCl₂, ZnCl₂, FeCl₃, AlCl₃, MnCl₂, and CoCl₂ was the same as that without the addition of metal ions (Figure 1). On the contrary, the addition of >0.1 mM NiCl₂ and CoCl₂ led to a lower growth level than no addition of metal ions at the first stage of cultivation (Figures 1A,B,K,L). Similarly, the growth level following the addition of ZnCl₂ and MnCl₂ was lower at 1 mM (Figures 1C,D,I,J). The growth trend did not change distinctly between 38 and 39°C (Figure 1). Ten millimolar CaCl₂ or 10 mM NaCl suppressed growth in the second step of *Z. mobilis* TISTR 548 cultivation at 38°C (data not shown). On the other hand, when MgCl₂ and KCl were added to the medium, there was a 1.3 higher growth than there was without adding metals even at 39°C (Figures 2A–C). At 39.5°C, there was negligible growth in the presence of MgCl₂ and KCl (Figure 2D).
The results suggested that the optimum concentrations of MgCl$_2$ and KCl for growth enhancement at 39°C were 5 and 30 mM, respectively.

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

Our previous report indicated that the cell length of *Z. mobilis* increased at CHT, and this increase reduced in thermotolerance-enhanced mutants (Kosaka et al., 2019). Indeed, cells grown at 39°C had longer cells than those grown at 30°C, which had granular shapes (Figures 3A,B). Cell morphology observed following the addition of MgCl$_2$ or KCl indicated that cell length increased, with the increase in cell length being relatively lower following the addition of Mg$^{2+}$ at 39°C than without the addition of metal ions (Figure 3C). On the other hand, the addition of KCl had no clear effect on cell length at 39°C, with a predominance of longer filamentous cells (Figure 3D). Cells cultured with both metals showed a mixture of granular and long filamentous shapes (Figure 3E). Indeed, the median value of measured cell length at 30°C, at 39°C, with MgCl$_2$ at 39°C, with KCl at 39°C, or with both metals at 39°C was 3.3, 7.6, 5.0, 7.2, or 6.1 µm, respectively (Figure 3F). Ethanol productivity at 39°C was also recovered to be close to the theoretical yield by adding MgCl$_2$ or KCl (Figure 3G). Accumulation of intracellular ROS was observed in *Z. mobilis* TISTR 548 at CHT (Kosaka et al., 2019). Addition of MgCl$_2$ or KCl considerably reduced intracellular ROS levels at 39°C, and the reduction strength was the highest for only K$^+$, followed by that for both metals and then only Mg$^{2+}$ (Figure 3H).
Effects of Mg$^{2+}$ and K$^+$ on Z. mobilis TISTR 548 Growth at CHT

Previous results indicated that Mg$^{2+}$ and K$^+$ somehow affect the cell physiology of Z. mobilis TISTR 548 at CHT and reduce intracellular ROS levels but probably by different mechanisms. Several bacteria use glutathione as a reducing agent to maintain a strongly reducing environment in cells, and glutathione peroxidase is an ROS-scavenging enzyme (Cabiscol Català et al., 2000). We observed the effect of glutathione with MgCl$_2$ or KCl on cell growth when glutathione was added at several concentrations: 4 mM glutathione inhibited cell growth at 39°C (data not shown) but 0.5 mM did not (Figure 4A). An Mg$^{2+}$ plus glutathione effect was observed, but the effect was not considerably distinct from that observed following the addition of K$^+$ or both metals (Figures 4B–D). Next, an effective concentration of EDTA as a chelator of a divalent cation on cell growth at a CHT were explored, and then effects of metals under the presence of such a concentration of EDTA at a CHT were examined. When 0.05 mM EDTA was added to culture, cell growth was inhibited at 38°C (Figure 5A). MgCl$_2$ or KCl was subsequently added under the above condition, and the resulting effect was observed. The addition of Mg$^{2+}$ rescued EDTA inhibition at CHT (Figure 5B), but that of K$^+$ did not (Figure 5C).

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

In a previous study, 26 thermosensitive single gene-disrupted mutants of Z. mobilis TISTR 548 were isolated (Charoensuk et al., 2017). To observe the effects of the addition of Mg$^{2+}$ and K$^+$ on these thermosensitive mutants, their growth upon the addition of each metal was examined. Addition of Mg$^{2+}$ significantly enhanced the growth of three mutants, whereas it inhibited the growth of one mutant (Table 2). Further, the addition of K$^+$...
TABLE 2 | Effects of Mg$^{2+}$ and K$^+$ on the growth of thermosensitive mutants from Zymomonas mobilis TISTR 548.

| Group                        | Tn10-inserted gene$^a$ | Strain | Function                          | Protein type | Growth$^b$ | Effect of MgCl$_2$$^c$ | Effect of KCl$^c$ |
|------------------------------|------------------------|--------|-----------------------------------|--------------|------------|------------------------|------------------|
| WT (TISTR 548)              |                        |        |                                   |              |            |                        |                  |
| General metabolism          | ZZ6_0707               | TC01   | Glucose sorboseone dehydrogenase  | Soluble      | ++++       | 108±0                  | 105±15           |
|                             |                        |        | +                                 |              | +          | 114±8                | 103±23           |
| Membrane stabilization      | ZZ6_1146               | TE12   | Glucosamine/fructose 6-phosphate aminotransferase | Membrane     | +          | 119±6    | 328±14            |
|                             |                        |        | −                                 |              | −          | 138±6                | 146±2            |
|                             | ZZ6_0923               | 3-24   | Glycosyl transferase group 1     | Soluble      | −          | 176±7    | 177±10            |
|                             |                        |        | −                                 |              | −          | 131±4                | 98±13            |
|                             | ZZ6_1477               | TC15   | Preprotein translocase subunit Tim44 | Membrane     | +          | 157±5    | 519±4             |
|                             |                        |        | −                                 |              | −          | 115±9                | 110±5            |
|                             | ZZ6_1058               | TC14   | Autotransporter secretion inner membrane protein TamB | Membrane     | +          | 118±9    | 165±10            |
|                             |                        |        | −                                 |              | −          | 112±5                | 223±7            |
|                             | ZZ6_1210               | C13-36 | Competence protein ComEC          | Membrane     | −          | 126±7    | 33±4              |
|                             |                        |        | −                                 |              | −          | 181±8                | 126±4            |
|                             | ZZ6_0840               | TC11   | Hypothetical transmembrane protein | Membrane     | −          | 116±5    |                  |
|                             |                        |        | −                                 |              | −          | 231±3                | 151±4            |
| Transporter                 | ZZ6_1289               | 1-10   | Putative Fe$^{2+}$/Mn$^{2+}$ transporter | Membrane     | −          | 112±5    |                  |
| DNA repair                  | ZZ6_0616               | F32    | DNA repair protein RadC           | Soluble      | ++++      | 113±9    | 96±4              |
|                             |                        |        | −                                 |              | +          | 156±6                | 146±1            |
|                             | ZZ6_0934               | C12-43 | Exonuclease VII (XseA)           | Soluble      | −          | 101±5    | 62±8             |
|                             |                        |        | −                                 |              | +          | 117±11               | 116±12           |
| tRNA/rRNA modification      | ZZ6_0681               | TC10   | DNA repair protein RadA          | Soluble      | +          | 153±5    |                  |
|                             |                        |        | −                                 |              | −          | 98±1                | 212±4            |
| Protein quality control     | ZZ6_1659               | C21-17 | Zn-dependent peptidase           | Soluble      | ++++      | 99±10    | 104±16           |
| Translational regulation    | ZZ6_0980               | TC05   | Serine protease DegP             | Soluble      | −          | 172±3    |                  |
|                             |                        |        | −                                 |              | −          | 172±3                |                  |
| Cell division               | ZZ6_0702               | TC12   | ATP-dependent helicase HrpB      | Soluble      | −          | 90±11               |                  |
| Transcriptional regulation  | ZZ6_0979               | TE19   | ParA/MnD-like ATPase             | Soluble      | −          | 327±2             |                  |
| Others                      | ZZ6_0962               | C12-37 | Pseudogene                       | Soluble      | +          | 161±9    |                  |
|                             |                        |        | −                                 |              | −          | 53±3                |                  |

$^a$Thirty-six thermotolerant genes were identified, and they were classified into 10 groups (Charoensuk et al., 2017).

$^b$The growth of these mutants at 38, 39, and 39.5°C compared with that of the parental strain on YPD medium. The symbols “+” represent the degree of cell growth of mutants at a critical high temperature compared with that of the parental strain, whereas “−” indicates no growth. The ratio (%) was calculated from the OD$_{492}$ values for cells grown at 39.5°C for 24 h with 5 mM MgCl$_2$ or 30 mM KCl divided by those cells grown without metals. Values represent means ± standard deviations of three replicates. Bold values indicate a ratio of >1.5 or <0.5 against the parental strain.

enhanced the growth of nine mutants, i.e., almost one-third of all thermosensitive mutants, but it inhibited the growth of one mutant (C13-36) at 39.5°C (Table 2). Remarkably, the addition of both Mg$^{2+}$ and K$^+$ enhanced the growth of only one mutant with gene encoding for phospholipase D; they had the opposite effect on the mutant TC13 (Table 2). These results also suggest...
that Mg\(^{2+}\) and K\(^{+}\) affect the cell physiology of *Z. mobilis* TISTR 548 differently at CHT.

**DISCUSSION**

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

Only Mg\(^{2+}\) and K\(^{+}\) enhanced *Z. mobilis* TISTR 548 growth and improved CHT from 38 to 39°C (Figure 2A). At CHT, the intracellular molecular components of bacterial cells leaked (Haught and Morita, 1966; Allwood and Russell, 1967). In *S. cerevisiae* and probably other microorganisms, the addition of K\(^{+}\) prevents ion leakage (Lam et al., 2014). K\(^{+}\) channels are activated when tension in the lipid bilayer is increased (Iwamoto and Oiki, 2018), and Mg\(^{2+}\) transporters are induced by heat treatment in *Salmonella enterica* (O’Connor et al., 2009). Regarding quantity, these two metal ions (K\(^{+}\) and Mg\(^{2+}\)) are the principal and second highest ions in bacterial cells found at concentrations of 100–500 mM (Ballal et al., 2007) and ~1 mM (Groisman et al., 2013), respectively. Therefore, the optimal concentrations of 30 mM K\(^{+}\) and 5 mM Mg\(^{2+}\) (Figures 2A,B) are probably related to their intracellular concentrations, further suggesting that similar ion conditions enhance cell metabolism by preventing ion leakage from cells or supporting ion transportation from the extracellular space. However, the effects of Mg\(^{2+}\) and K\(^{+}\) on the two common characteristics of bacteria, namely, cell elongation and ROS accumulation, observed in *Z. mobilis* TISTR 548 at CHT (Matsushita et al., 2016) were different. Cell length at CHT was suppressed by the addition of Mg\(^{2+}\) but not by that of K\(^{+}\) (Figure 3F). Although ROS accumulation reduced by the addition of both metals, the addition of K\(^{+}\) showed a stronger effect than that of Mg\(^{2+}\) (Figure 3H). The GSH results indicated that the additive effect of GSH was observed in both cases of Mg\(^{2+}\) and K\(^{+}\) (Figures 4B,C), suggesting that the growth enhancement effect of Mg\(^{2+}\) or K\(^{+}\) does not arise directly from the action of GSH added exogenously. In a Gram-negative bacterium, *E. coli*, GSH is important for periplasmic redox homeostasis (Pittman et al., 2005) and heterogeneous expression of glutathione reductase allows the microbe to be hydrogen peroxide tolerance (Kim et al., 2009). It is assumed that, in *Z. mobilis* TISTR 548, GSH keeps periplasmic redox homeostasis and/or somehow makes cells tolerate oxidative stress by its reducing power, but the major effects at CHT by Mg\(^{2+}\) and K\(^{+}\) are not likely the action by GSH. Moreover, EDTA treatment showed that K\(^{+}\) did not complement the EDTA effect at CHT (Figure 5C). These results suggest that Mg\(^{2+}\) and K\(^{+}\) affect the cell physiology of *Z. mobilis* TISTR 548 at CHT using different mechanisms.

The effect of Mg\(^{2+}\) on the cell physiology of *Z. mobilis* TISTR 548 at CHT has been described: Mg\(^{2+}\) probably stabilizes membrane structure as proposed in *E. coli* (Charoenruk et al., 2017). Mg\(^{2+}\) stabilizes OM (Nikaido and Vaara, 1985), particularly LPS, where Mg\(^{2+}\) bridges lipid A (Nikaido, 2003). The present study results also showed that the addition of Mg\(^{2+}\) repressed cell elongation at CHT (Figure 3F) and restored the growth of the disrupted genes of ZZ6_0923, which encodes the cardiolipin biosynthesis protein (Table 2). However, Mg\(^{2+}\) has been thought to stabilize proteins, enhance protein–nucleic acid interactions, mitigate oxidative stress, and act as a metabolic signal (O’Connor et al., 2009). Mg\(^{2+}\) is required...
to maintain cell metabolism, DNA replication, transcription and translation, and DNA stabilization (Xu et al., 2018), and it plays a role in enzyme activations. For instance, Mg$^{2+}$ stabilizes pyruvate decarboxylase, an enzyme responsible for the decarboxylation of pyruvate in central metabolism, with thiamine diphosphate serving as a cofactor (Pohl et al., 1994). Besides, phosphoglycerate kinase uses Mg$^{2+}$ as a cofactor (Andreini et al., 2008). Addition of Mg$^{2+}$ reduced ROS accumulation at CHT (Figure 3H), and the growth of the disrupted Fe$^{2+}$/Mn$^{2+}$ transporter (ZZ6_1289) recovered greatly (Table 2). Therefore, maintaining an intracellular Mg$^{2+}$ concentration may enable heat tolerance either by ions or cytoplasmic Mg$^{2+}$ sensors, proteins, and RNAs. (Groisman et al., 2013).

K$^+$, the most dominant intracellular cation, greatly contributes to pH homeostasis and turgor maintenance as well as bacterial osmotic adaptation, pH regulation, gene expression, and cell enzyme activation (Epstein, 2003; Ballal et al., 2007). Indeed, the addition of K$^+$ affected most cell physiology of Z. mobilis TISTR 548 at CHT (Table 2), e.g., the growth of 35% (9/26 strains) of mutants recovered greatly. These effects may contribute to reducing intracellular ROS levels (Figure 3H). Between these, K$^+$ probably facilitates the functioning of periplasmic proteins in Z. mobilis TISTR 548 due to the growth recovery of disrupted tolQ, secD, tamB, and degP (Table 2). The amount of intracellular K$^+$ directly affects membrane potential (Bakker and Mangerich, 1981), which is required for protein secretion to periplasm (Daniels et al., 1981). The membrane potential is hypothetically required for potassium transport from extracellular space to the cytoplasm via the membrane potential-driven K$^+$ uptake system (Kup, ZMO1209, and ZZ6_0125) in Z. mobilis. Therefore, K$^+$ may also facilitate membrane potential maintenance in Z. mobilis TISTR 548.

Under CHT conditions (Figure 6A), the inner membrane fluidity increases to cause leakages of ions from cytoplasm and electrons from the respiratory chain, which lead to the accumulation of intracellular reactive oxygen species, resulting in damage of macromolecules of DNA, RNA, proteins and lipids, and thereby cells are elongated and unable to maintain intracellular homeostasis, causing cell death. However, by the addition of Mg$^{2+}$ (Figure 6B), the OM is stabilized by binding of Mg$^{2+}$ and the inner membrane is also stabilized, resulting in suppression of the leakage of intracellular ions as well as the leakage of electrons from the respiratory chain. On the other hand, by the addition of K$^+$ (Figure 6C), K$^+$ leakage is repressed to maintain homeostasis for cellular metabolism, by which intracellular ROS is reduced. Moreover, these observations suggest that Mg$^{2+}$ and K$^+$ exhibit diverse, rather than single, effects on Z. mobilis TISTR 548. Interestingly, when both Mg$^{2+}$ and K$^+$ exist in the medium at high concentrations, their crosstalk effects on cell physiology sometimes occur. These effects are partly specific to each ion; their additive effect on cell growth at CHT was observed, but that did not entail the whole sum of their effects (Figure 2C). The thermotolerance acquisition mechanisms of Z. mobilis upon the addition of Mg$^{2+}$ and K$^+$ are more complex than the accumulated effects of their metals in accomplishing enhanced Z. mobilis growth at CHT.

CONCLUSION

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

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/supplementary material.

AUTHOR CONTRIBUTIONS

AN, TS, KM, and SA conducted the experiments. TK, AN, TS, KM, and MY analyzed the data. TK, AN, and MY wrote the manuscript. All authors conceived this study.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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