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学 位 論 文 要 旨
(Summary of the Doctoral Dissertation)

学位論文題目 (Dissertation Title)	Improvement of The Heat Resistance Ability of Thermotolerant *Zymomonas mobilis* TISTR 548 （耐熱性 *Zymomonas mobilis* TISTR 548 の耐熱性の改良）
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Fundamentally, microorganisms have an upper-temperature limit of survival that is called a critical high temperature (CHT) (Matshushita *et al.*, 2015; Kosaka *et al.*, 2019), which may be a combined results of various cellular elements including general and particular metabolisms. Mesophilic microbes that have a CHT close to the range of global warming temperatures would be damaged by an increase in temperature. Thermotolerant microorganisms are able to grow and/or perform fermentation at higher temperatures than those for mesophilic microorganisms. In this study, two experiments on the enhancement of thermotolerance of *Z. mobilis* were carried out. One experiment was on improvement of thermotolerance of *Zymomonas mobilis* by genes for reactive oxygen species (ROS)-scavenging enzymes (RSEs) and heat shock proteins (HSPs), and the other experiment was on improvement of the thermotolerance of *Z. mobilis* by metal ions.

A temperature close to the critical high temperature (CHT) influences membrane stability and generates ROS, resulting in macromolecule damage such as protein unfolding or denaturation. However, in mesophiles, including *Z. mobilis,* only a few genes for ROS-scavenging enzymes (RSEs) and heat shock proteins (HSPs) have been identified as thermotolerant genes that are essential at the CHT. We attempted to increase the expression of genes for RSEs (*sod, cat, cyt, ZZ6_1529, ahpC1, ahpC2,* and *ZZ6*_0186) and genes for HSPs (*degP, dnaK, dna J, groEL, groES, hslU, ibpA, clpA, clpB, clpS, and ZZ6*_0844). The influence of these genes at the CHT of *Z. mobilis* TISTR 548 was evaluated by the 2-step cultivation method, determination of ROS and cell length, and

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examination of resistance to other stresses. The results showed that increased expression of genes for RSEs and HSPs led to an up-shift of the CHT of *Z. mobilis* TISTR 548, though the range of increase in the CHT was only within 1°C, and a decrease in the accumulation level of ROS. However, among the genes for HSPs that were tested, the genes *degP, hslU* and *hsp33* were unable to raise the CHT when their expression was increased, even though they were able to decrease the accumulation level of ROS to some extent. Examination of the effect of oxidative stress on transformants by addition of H_2O_2 to the culture medium showed that the conclusion was essentially consistent with the results of measurement of levels of ROS. The change of cell morphology was also relatively consistent with the effects of the genes on cell growth at the CHT. Moreover, when glucose or ethanol was added to the culture medium, none of the transformants that had any gene for RSEs and HSPs showed that was different growth from that of the control. However, some of the transformants showed enhanced tolerance to acetic acid. These findings suggest that molecules damaged by ROS or unfolded proteins that prevent cell growth accumulate in cells at the CHT. Therefore, prevention of the accumulation of ROS might be effective way for increasing the CHT. The expression of genes for RSEs is thought to play a direct role in prevention of the accumulation of ROS, whereas the expression of genes for HSPs is assumed to play an indirect role by maintaining the quality of proteins including RSEs, which in turn helps to prevent accumulation of ROS and increase heat resistance capability.

Furthermore, with the aim of improving the ability for thermotolerance, we investigated the effects of various metal ions on growth of *Zymomonas mobilis* TISTR 548 at the CHT. Addition of Mg^{2+} and K^{+} clearly improved growth by 1°C, but Mn^{2+}, Ni^{2+}, Co^{2+}, Al^{3+}, Fe^{3+}, and Zn^{2+} showed negligible effects. These results indicate that only specific metals are required for the enhancement of growth of specific microorganisms. To understand the physiological functions associated with the addition of Mg^{2+} or K^{+}, cell morphology, intracellular level of ROS, and ethanol productivity were investigated at

39°C. Cell elongation was repressed by Mg^{2+}but not by K^{+}. The greatest reduction in intracellular levels of ROS was achieved by addition of only K+, followed by both metals and addition of only Mg^{2+}. Additionally, ethanol productivity was recovered by the addition of both metals. Moreover, the addition of Mg^{2+} or K^{+} at a nonpermissive temperature to cultures of 26 thermosensitive, single gene-disrupted mutants of *Z. mobilis* TISTR 548 resulted in improvement of metal ion-specific growth of several mutants. Remarkably, K^{+} repressed the growth of two mutants. These results suggest that K^{+} and Mg^{2+} enhance cell growth at the CHT via different mechanisms, which are involved in the maintenance of low intracellular levels of ROS. For more details, under a CHT condition, the addition of Mg^{2+} resulted in stabilization of both the outer and inner membranes, and leakage electrons from respiratory chain was suppressed. Membrane stabilization also helps to maintain intracellular ions, cytoplasmic sensors, proteins, and RNAs. On the other hand, when the K^{+} was added, the repression of K^{+} leakage capable to maintain the homeostasis for cellular metabolism whereby intracellular ROS is reduced. However, the observation of combinative usage of Mg^{2+} and K^{+} exhibits a distinct result compares to the single effect.

In conclusion, the results of both of the studies described above suggest that there are two ways to address the problem of accumulation of ROS at a CHT: (1) suppression of the generation of ROS and (2) elimination of accumulated ROS. In fact, increased expression of genes for RSEs suppressed the generation of ROS and improved growth at high temperatures, thus increasing the CHT. On the other hand, Mg^{2+} may have an effect on accumulation of ROS by mainly blocking the generation of ROS preventing electron leakage through strengthening the membrane structure. The actions of the increased expression of genes for HSPs and addition of K^{+} appear to improve thermostability in either or both of these two ways. It is possible that the combination of these genes and metals greatly improves the thermostability of *Z. mobilis*. Therefore, further study on stable high temperature fermentation is needed.

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学位論文審査の結果及び最終試験の結果報告書

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【論文審査の結果及び最終試験の結果】

令和２年８月７日（金）、上記審査委員６名で構成された審査委員会は、山口大学において、学位申請者本人に研究内容を公開発表会の形式で発表させ、その内容について質疑応答を行った。また関連事項についても試問を行った。その結果、研究内容はオリジナリティが高く、その成果は関連分野の水準を高めるものであり、また質問に対して満足すべき回答が得られた。

本研究は、エタノール生産菌*Zymomonas mobilis*について耐熱性の向上と耐熱性機構の解明を目的として2つの研究を実行したものである。生育限界温度（CHT）で活性酸素種（ROS）が蓄積することから、1つ目の研究は、ROS消去酵素（RSE）と熱ショックタンパク質（HSP）の遺伝子導入、2つ目は、金属イオン添加の影響を検討したものである。前者では、CHTにおいてHSP遺伝子の一部を除いた遺伝子導入によって、後者では、Mg^{2+}とK^{+}添加によってROS蓄積量が減少し、耐熱性が強化された。それらの結果から、RSE遺伝子導入はROS除去によって、Mg^{2+}は膜構造の強化によるROSの生成抑制によって高温での成長を改善すると推測される。一方、HSP遺伝子導入とK^{+}の添加の効果は、より複雑で、特にK^{+}は細胞内の恒常性維持が考えられる。このように、単に耐熱性強化だけでなく、CHTにおける細胞膜や代謝の変化の理解につながる研究として高く評価される。

よって、審査委員会は同人を山口大学大学院創成科学研究科博士後期課程修了者として相応しい学力と識見を有するものと認め、博士（生命科学）の学位を与えるに十分な資格を有するものと判定した。