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SUMMARY OF DOCTORAL THESIS

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Title: Molecular biological study on alcohol dehydrogenases in ethanologenic thermotolerant yeast *Kluyveromyces marxianus*
エタノール生産性の耐熱性酵母 Kluyveromyces marxianus のアルコール脱水素酵素の分子生物学的研究

Alcohol dehydrogenases (Adhs) (E.C. 1.1.1.1) are enzymes present in almost all organisms, Pincluding bacteria, yeast, insects, mammals, and plants, and they participate in ethanol production or ethanol degradation. Adhs are highly conserved in the primary sequence in yeast, but the number and expression patterns of their genes might be different. In *Saccharomyces cerevisiae* and *Kluyveromyces lactis,* seven and four *ADH* genes have been cloned and identified, respectively.

Kluyveromyces marxianus is a yeast currently being investigated as an alternative to *S. cerevisiae* for utilization in ethanol production. It has three promising properties: ability to grow at relatively high temperatures, growth on a great variety of inexpensive carbon sources, and short doubling time. Therefore, potential biotechnological applications of *K. marxianus* strains are manifold. However, there is a lack of information in the literature concerning its physiology. In order to establish a method of ethanol production at high temperatures, which is expected to reduce the cost of cooling, knowledge of the physiology of Adh enzymes may be indispensable.

In the present study, four genes encoding alcohol dehydrogenase (Adh) isozymes in the thermotolerant yeast *K. marxianus* were cloned and investigated. The four KmAdhs had high sequence similarity, though KmAdh3 and KmAdh4 possessed an amino-terminal extension as a mitochondrial targeting sequence. They appear to belong to the zinc-containing Adh family. These and the results of Southern blot analysis suggest that there are at least four Adh isozymes in *K. marxianus*, two cytoplasmic enzymes and two mitochondrial enzymes. The cytosolic KmAdh1 and KmAdh2 may be responsible for the production of ethanol during glucose fermentation, but physiological roles of mitochondrial KmAdh3 and KmAdh4 remain to be elucidated. *KmADH3* is expressed on respiratory carbon sources including glycerol. In contrast, the expression level of *KmADH4* is high on ethanol but low on glycerol. The both are hardly

expressed on glucose. Two mitochondrial Adhs in *S. cerevisiae* and *K. lactis* also appear to be under the specific regulation depending on carbon sources. Additionally, the expression of *KlADH3* might be related to that of genes for succinate dehydrogenase. Therefore, they are thought to have distinctive contributions to mitochondrial metabolism, but the evidence on their functions is still very limited.

Here, the function of mitochondrial Adh3 in thermotolerant yeast *Kluyveromyces marxianus* was investigated. An *ADH3*-disrupted mutant exhibited growth retardation on non-fermentable carbon sources except for ethanol, which was suppressed by supplementation with antioxidants. Detailed analysis on the phenotype revealed that the mutant showed increase in activity of NADH dehydrogenase, sensitiveness to H_2O_2 and accumulation of reactive oxygen species (ROS), and that these carbon sources increased in activity of succinate dehydrogenase. The increase in both activities may reflect enhanced expression of both dehydrogenases by elevation of their substrate levels. The ROS level became low when antioxidants were added. These findings suggest that *ADH3* mutation and such carbon sources cause in elevation of the substrate level of the respiratory chain and eventually of ROS level via increased expression of primary dehydrogenases, which in turn causes cell growth retardation. Adh3 may thus play a crucial role in the control of NADH/NAD^+ balance in mitochondria.