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SUMMARY OF DOCTORAL THESIS

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Title: **Studies on 5-keto-D-gluconic acid (5KGA) production at higher temperature by *Gluconobacter* strains: screening of thermotolerant strains and development of high 5KGA-producing strains**

グルコノバクター属酢酸菌による高温 5-ケトグルコン酸（5KGA）生産系に関する研究：耐熱性酢酸菌のスクリーニングと高温 5KGA 生産菌の開発

Gluconobacter is a genus of acetic acid bacteria which are able to oxidize a broad range of sugars, sugar alcohols and sugar acids, and which accumulate large amounts of the corresponding oxidized products in their culture medium. The production of ketogluconates by *Gluconobacter* strains has been reported to be catalyzed by two types of membrane-bound gluconate dehydrogenases. One is FAD-gluconate dehydrogenase (FAD-GADH), an FAD-containing, 2-keto-D-gluconate (2KGA)-yielding enzyme, and the other is PQQ-glycerol dehydrogenase (PQQ-GLDH), a PQQ-containing, 5-keto-D-gluconate (5KGA)-yielding enzyme. In this study, in order to have selective 5KGA production system able to be done without serious temperature control, high 5KGA-producing strain under higher temperature was developed.

For simple and precise measurement of 5KGA and 2KGA, 5KGA reductase (5KGR) and 2KGA reductase (2KGR), present in the cytoplasm of *Gluconobacter*, are useful, and thus purified in this study. Since the gene for 5KGR has been reported but the gene for 2KGR never, the 2KGR gene was identified in this study. Two plasmids were constructed to express *GOX2187* (5KGR gene) and *GOX0417* (2KGR) gene in *Escherichia coli*. By using the two *E. coli* transformants, large amounts of each enzyme were purified after a single column chromatography, and thus the enzyme assay system for 5KGA or 2KGA was established.

Thermotolerant *Gluconobacter* strains able to produce 5KGA at 37 °C were isolated, which were superior to the mesophilic strains which could not produce 5KGA at such a high temperature. Thermotolerant *Gluconobacter frateurii* THE42, THF55, and THG42 were selected and shown to accumulate 2KGA and 5KGA as the major and minor products, respectively, at both 30 and 37 °C.

In order to develop high 5KGA-prodcer, 2KGA-producing GADH was tried to be disrupted. At first, the gene for FAD-GADH in the thermotolerant strains, *gndFGH*, was cloned by inverse PCR and shown to be present in all the isolated themotolerant strains (THE42, THF55, and THG42). Disruption of *gndG* was carried out in all the three strains and thus the disrupted mutants were shown to almost exclusively produce 5KGA at 30 °C, although the 5KGA production was decreased at 37 °C, it was found that the addition of calcium chloride in the culture medium increased 5KGA production even at 37 °C. Thus, the newly developed strains provide an alternative method for 5KGA production at higher temperatures.

In *G. dioxyacetonicus* IFO 3271 (reclassified as *G. frateurii* NBRC 3271), FAD-GADH gene, *gndSLC*, having low homology to *gndFGH*, has been cloned and the disruption of *gndL* has been shown not to abolish 2KGA production, although the production significantly decreases, concomitant with the decrease in GADH activity. Thus, it has been expected that another 2KGA-yielding enzyme should be present in this strain. In this study, the additional GADH gene was isolated by inverse PCR. Six open reading frames were found and 3 of them showed high identities to *gndFGH*. Thus, *G. frateurii* NBRC 3271 was shown to have two 2KGA-yielding enzymes, GADH-1 encoded by *gndSLC* and GADH-2 encoded by *gndFGH*. When different GADH mutants were constructed from the wild-type strain of *G. frateurii* NBRC 3271, *gndG* mutant exhibited the production of 2KGA similar to the wild-type, whereas *gndL* mutant produced lower amounts of 2KGA with higher 5KGA production comparing to the wild-type. The double disruptant mutant almost completely produced 5KGA with trace amounts of 2KGA that would be produced from the 2KGR in cytoplasm. These results confirmed that *gndG* and *gndL* are responsible for 2KGA production in this strain.