Doctoral Thesis

Isolation of *Halomonas* strains from Food-related environments and development of whole genetic tools for *Halomonas* sp. KM-1, a promising bacterium for biochemical production.

Major of Biotechnological Science,
Graduate School of Biology-Oriented Science and Technology,
Kindai University

Ayaka Tsuji

SUMMARY

Halomonas species, which are aerobic, alkaliphilic, and moderately halophilic bacteria, are often isolated from high saline environment such as the seas and salterns. They are promising for industrial productions of diverse biochemicals, such as polyhydroxybutyrate and ectoine, by fermentation due to their magnificent abilities including high-density culturing capacity and low-risk contamination. This is a study on *Halomonas* in isolation of novel useful bacteria from food-related environment in Japan, physiological understanding of *Halomonas* sp. KM-1 (KM-1) which is being used as a useful bacterium, and establishment of essential techniques on metabolic engineering. This study was consisted of 4 sections, and in the introductory chapter, the substance productivity of KM-1 which already reported was reconfirmed. KM-1 was isolated from the soil in Osaka, and reported to highly produce pyruvate, polyhydroxybutyrate (PHB) and 3-hydroxybutyrate (3HB), a monomer of PHB. Since 3HB has various physiological activities such as suppression the growth of the cells and arteriosclerosis, it is promising for application to pharmaceuticals and supplements. However, there are few reports of *Halomonas* isolation in Japan besides KM-1. As isolation of *Halomonas* progressed in many areas of the world, it is a big issue even in Japan to understand habitat areas of *Halomonas* and secure the *Halomonas* strains which can be used for substance production.

In the introductory chapter, follow-up study suggested that temperature and carbon source have a great influence on pyruvate production. It implies the possibility that the productivity exceeds the one published in the paper, and there is room to dramatically improve it by genetic modification. On the other hand, proteome analysis of KM-1 during substance production is conducted to compare and analyze the states of protein accumulation. A hemolysin coregulated protein (Hcp), an effector protein of type VI secretion system, was highly and constitutively accumulated, and a promoter of its gene was used for genetic engineering in the chapter II. In the genome of KM-1, there are some genes cording phasin which is located on surface of PHB granules and intermediate their stabilization. One of the phasins highly accumulated in a stationary phase and

another phasin was degraded in a 3HB secretion phase. In KM-1, no one studied the physiological analysis on PHB production including phasin, and the function of these phasins were completely unknown, which also indicated the room for dramatic improvement in the productivity by genetic engineering. The lack of the established genetic tools to analyze the functions of KM-1 and improve the productivities is an extremely big problem to be solved.

In the chapter I, isolation of *Halomonas* strains from food-related resources was attempted to understand the habitat of *Halomonas* in Japan, which is the issue presented in the first half of the introductory chapter. As a result, 20 *Halomonas* strains were successfully isolated from edible seashells, a shrimp, and umeboshi factory effluents. It was revealed that there are strains in the nature world which are as useful as KM-1 in terms of 3HB productivity, salt tolerance, proliferable pH and temperature. We have also discovered a strain that produces ectoine, which is used as an important moisturizer for pharmaceuticals and cosmetics. On the other hand, isolates from umeboshi factory effluents harbored plasmids and whole-genome sequencing of *Halomonas* sp. A020 (A020) harboring 2 plasmids was performed to determine its origin and genomic characteristics. The DNA sequences of the plasmids were determined, and the regions of replication *ori* of the plasmids were used for development of genetic tools for KM-1 in the chapter II.

In the chapter II, shuttle vectors which can be used for KM-1 were constructed to develop genetic tools for *Halomonas*, which was the subject presented in the latter half of the introductory chapter. As the replication *ori* of these shuttle vectors, the region of replication *ori* of the 2 plasmids in A020 appeared in the chapter I were used, 2 different shuttle vectors were constructed, and stable co-existence of the vectors in KM-1 was confirmed to be possible. It was also indicated that the IPTG inducible *E. coli* promoter was available and the promoter of Hcp, which was highly accumulated protein appeared in the introductory chapter, was able to highly express the genes. Moreover, in order to utilize the CRISPR-Cas9 system in genetic engineering of KM-1, *cas9* gene and guide

RNA were inserted to each constructed shuttle vector, and expression of *cas9* gene and guide RNA were confirmed. In the CRISPR-Cas9 introduced KM-1, disruption of *pyrF* gene, which is a homolog of yeast *URA3* gene and important in pyrimidine synthesis, was attempted. Some *ΔpyrF* mutants were obtained, but many of them had mutations in off-target sites in *pyrF* gene. However, no *ΔpyrF* mutants was obtained in a control, and it is suggested that mutations were occurred depend on the CRISPR-Cas9 system. Furthermore, the gene disruption in this study indicated that all strains except one had short DNA defects. There were repeat sequences (microhomologies) with a length of several bases at the ends of the DNA-deficient region, and the identity with the mechanism of the CRISPR-Cas9 system reported in the other paper was confirmed. It is expected to improve the physiological understanding and substance productivities of *Halomonas* by engineering the metabolic regulations in *Halomonas* using the developed genetic tool.

In the chapter III, obtaining the central metabolic mutants was attempted to improve the substance productivities of KM-1 indicated in the introductory chapter and promote understanding of the metabolic regulations. From the UV irradiation mutants of KM-1, mutants which couldn't utilize glucose or citrate were screened. As a result, NO542, a citrate auxotroph which couldn't utilize glucose, and NO544, a glucose one which couldn't do citrate showing the opposite phenotype, were obtained. Based on the profile of carbon source utilization, it was predicted that NO542 was a mutant in glucose metabolism and NO544 was one in a citrate transporter. Whole genome analysis revealed that NO542 and NO544 have mutation in the HKM1_3652 gene of the pentose phosphate pathway and the HKM1_1402 gene annotated as a Na^+/H^+ antiporter, respectively. Each mutation was complemented by introducing the HKM1_3652 gene and the HKM1_1402 gene from the wild-type strain, and the mutant genes were identified. Analysis of the NO542 mutant indicated the importance of not only glycolysis but also the pentose phosphate pathway in glucose metabolism in KM-1 and provided important insights into the modification of the metabolic pathway. Analysis of the NO544 mutant revealed that

the gene, which was annotated as a Na^+/H^+ antiporter in numerous species, functioned as a sodium dependent citrate transporter.