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論文内容の要旨

Sirtuin, often referred as the longevity gene, was first identified as SIR2 in yeast and its homologous genes have been widely found in both eukaryotic and prokaryotic organisms. In addition, the main enzyme activity of sirtuin proteins were proven that it was a NAD^+-dependent deacetylase of which the substrates are histones, p53 and other acetylated proteins in the nucleus or cytoplasm. In prokaryotes, sirtuin gene was identified as *cobB*, encoding a cobalamin processing enzyme, and later its involvement in regulating metabolic enzymes, transcription factors, chemotactic proteins and others as NAD^+-dependent deacetylase. On the other hand, little is known about its roles in lactic acid bacteria (LAB).

LAB are widely consumed by human as food fermentation starters and as probiotics. Probiotics are live bacteria that are thought to be beneficial in preventing several health conditions. According to the 2002 definition by the World Health Organization (WHO), probiotics are "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host." Therefore, those probiotics bacteria must be alive in probiotics product before they were administered to the host. In addition, the important function of the probiotics was known to regulate the functions of intestine and immunopotentiative actions. Therefore, the author assumed that it is important to increase stress tolerance, intestinal tract adhesion and the useful substance produced by LAB in order to confer more beneficial health effects on the host. In this study, the author intended to analyze the role of sirtuin in LAB.

In the chapters 1 and 2, the author analyzed sirtuin homolog genes of LAB and analyzed enzyme kinetics of the recombinant *L. paracasei* sirtuin proteins. In the chapter 3, the author identified several candidate target proteins both *in vivo* and *in vitro* by using the recombinant LpSirA protein and sirtuin inhibitor nicotinamide (NAM). One of the target was identified as 30S ribosomal protein S4. In the chapter 4, the author analyzed intracellular localization of sirtuin using immunofluorescent staining and LpSirA-Venus fusion protein in *L. paracasei*.

Chapter 1. Identification of sirtuin genes in the genomes of the *L. paracasei* strains and its homology to sirtuins of related bacteria

The author demonstrated that almost all LAB have homologs of eukaryotic sirtuin. Interestingly, the author was not able to find the gene in *Lactococcus* spp and *Melissococcus* spp. In a previous study, the author's group determined draft genome sequences of three *L. paracasei* strains NRIC 0644, NRIC 1917 and NRIC 1981 (Shiwa et al., 2015). The sirtuin genomes of three strains identified highly homologous (99.9%) to those of *L. paracasei* BL23. The author tentatively designated the gene as *sirA* (indicating the first sirtuin homolog of *L. paracasei*). Interestingly, there was another sirtuin isozyme found in the genome of strain NRIC 1981, which was hit by BLAST for *L. rhamnosus* GG sirtuin with 78% identity. We designated this second gene as *sirB*, which was not found in the ge-

nomes of the strains NRIC 0644, and NRIC 1917. LpSirA encoded by *sirA* (693 bp) gene was shown to have two conserved active histidine residues and the NAD^+-binding motif as reported in human SIRT1. On the other hand, the LpSirB encoding by *sirB* (726 bp) gene contained similar NAD^+-binding domains, but only one conserved active histidine residue (his 79) site. When comparison was made using LALIGN program, the LpSirA sequence was 26.2% homologous to yeast Sir2 (BL23 LpSirA residues 8–226 vs *S. cerevisiae* Sir2 residues 251–518) and 29.4% homologous to human SIRT1 (BL23 LpSirA residues 3–208 vs SIRT1 residues 245–469). The amino acid sequence homology of LpSirA proteins with sirtuins of *E. coli*, *Salmonella enterica* serovar Typhimurium LT2 and *B. subtilis* were 29.3%, 29.5% and 31.1% respectively. The amino acid sequence of LpSirB protein showed only 80% homology to other reported *L. rhamnosus* sirtuins which share 94–97% homology with each other.

Chapter 2. Enzyme kinetics of the recombinant LpSirA protein

Expression and purification of recombinant *L. paracasei* sirtuin was conducted with *E. coli* expression system. The *sirA* and *sirB* were cloned into bacterial protein expression vector pET-15b. The constructed pET-15b plasmid was transformed into *E. coli* BL21 (DE3) to express recombinant LpSirA and LpSirB. The recombinant proteins with histidine tag were expressed and purified on a Nickel-affinity column. However, the recombinant LpSirB protein was found in the inclusion body of *E. coli* BL21 (DE3). Therefore, for the purification, the protein was dissolved in the presence of 8 M urea. Recombinant proteins were detected in single bands as apparent molecular sizes of 29 kDa for LpSirA, and 34 kDa for LpSirB on SDS-PAGE.

Kinetics studies were performed using *Flour de Lys*® fluorimetric activity measurement kit. The relative fluorescence unit was measured using NanoDrop 3300 Fluoropectrometer. The substrate used was an acetylated peptide comprising amino acids 379–382 of human p53. The control recombinant SIRT1 protein was provided in the same kit. The apparent Km and Vmax of SIRT1 and LpSirA from NRIC 0644, 1917 and 1981 were calculated from Line weaver Burk plot obtained in the presence of 3 mM NAD^+ (fixed) and 0.2 μg of each enzyme protein in the reaction mixtures. The standard curve was calculated from using deacetylated fluorescent peptide substrate (*Flour de Lys*® deacetylated standard) provided in the kit. In addition, the author tested SIRT1 activator resveratrol and SIRT1 inhibitor suramin. The apparent Km values were determined to be 130.2 μM, 186.3 μM, 180.1 μM and 130.1 μM for human SIRT1, *L. paracasei* NRIC 0644 LpSirA, NRIC 1917 LpSirA and NRIC 1981 LpSirA, respectively. The Vmax values were 257.5 nmol/min/mg, 160 nmol/min/mg, 170 nmol/min/mg and 212.5 nmol/min/mg for SIRT1, NRIC 0644 LpSirA, NRIC 1917 LpSirA and NRIC 1981 LpSirA proteins, respectively. The optimal temperature for the enzymatic reaction of LpSirA proteins displayed higher optimal temperature (45–50℃) than SIRT1 (37℃). In addition, Resveratrol decreased in the apparent Km values of human SIRT1, but the conclusion has not been reached yet on the resveratrol effects to apparent Km values of NRIC 0644 LpSirA. Therefore, in addition to examine the reproducibility, the author thinks it is important to search for new activator which strongly activate the enzyme activity of LpSirA. Suramin inhibited deacetylase activity of both human SIRT1 (IC_{50} : 18 μM) and NRIC0644 LpSirA (IC_{50} : 359 μM). The results suggests the similarity and the difference of enzyme property between human SIRT1 and LpSirA.

Chapter 3. Identification of the sirtuin-target acetylated proteins in *L. paracasei* BL23

Hereafter, instead of the three strains, a widely studied standard strain, *L. paracasei* BL23 was used. The putative target proteins of sirtuin in *L. paracasei* BL23 were first screened by inhibiting sirtuin deacetylase using NAM in the culture medium. In parallel, cell extracts (from cells cultured in MRS supplemented with 50 mM NAM) containing 100 μg protein was treated *in vitro* with 10 μg purified recombinant LpSirA in the presence of 10 mM NAD^+ to maximize NAD^+-dependent deacetylation. From each of the *in vivo* and *in vitro* target samples obtained above, 100

μg cellular protein was subjected to 12.5% SDS-PAGE, and blotted onto PVDF cellulose membrane. Western blotting was done using anti-LpSirA primary antibody or Acetylated-Lysine primary antibody, together with donkey anti-rabbit IgG secondary antibody. The results from each of the *in vivo* and *in vitro* samples indicated that a 28 kDa acetylated protein was the target protein of sirtuin. Furthermore, the 28 kDa acetylated protein was purified using ammonium sulfate precipitation (0–80%), Butyl-Toyopearl column and DE52 column. The elution at 0.5 M NaCl from DE52 column showed single band of an acetylated 28 kDa protein, and this protein was deacetylated by LpSirA. The fraction containing 28 kDa protein was further concentrated by acetone precipitation (80%), and subjected to SDS-PAGE followed by electroblotting onto FluoroTrans® PVDF Transfer Membrane. Its N-terminal amino acid sequences were determined by the Edman degradation method using a peptide sequencer PPSQ30. The N-terminal Amino acid sequence of the 28 kDa target protein was determined to be SRYTGPRWKQ, which was perfectly identical to that of 30S ribosomal protein S4 of *L. paracasei* in the data bank. The function of 30S ribosomal protein S4 was known to assemble the rRNA together with 30S ribosomal subunit protein S5 and S12. Additionally, the ribosomal large subunit component MRP10 of mitochondria was deacetylated by mitochondrial sirtuin (SIRT3), and it was found to decrease protein synthesis rate. Thus, the author presumed that LpSirA is involved in the regulation of protein synthesis in *L. paracasei* BL23.

Chapter 4. Intracellular localization of sirtuin protein in *L. paracasei* BL23

First, the author analyzed intracellular localization of sirtuin using anti LpSirA antibody in *L. paracasei* BL23. Cells were fixed on cover glasses and treated with 4% paraformaldehyde for 20 min. Cells were probed with a rabbit anti-LpSirA antibody (1 : 250 dilution) and anti-rabbit Alexa Fluor® 488 dye antibody (1 : 2,000 dilution) for 1 h at 37℃. The nucleoid of the cells were stained with 5 μg/ml Hoechst 33342 for 10 min. Finally, a drop of SlowFade® Diamond was applied to the sample and localization of LpSirA was examined in *L. paracasei* BL23 by Confocal Microscopy. The result showed that LpSirA was localized on the division plates and cellular poles during cell division.

Next, the author analyzed intracellular localization of sirtuin using LpSirA-venus fusion protein in the living cells of *L. paracasei* BL23. The *sirA* gene sequence (693 bp) was cloned into pCS2 Venus plasmid equipped with an incorporated Venus gene. The *sirA*-venus fusion gene was cloned into bacterial protein expression vector pLPM11. The pLPM11 construct (5 μg) was transformed into competent *L. paracasei* BL23 cells by electroporation. Transformant cells expressing LpSirA-Venus were inoculated into 5 ml MRS medium containing erythromycin and were grown for 12 hours at 37℃ in incubator. The 5 ml culture was washed with 0.86% saline and inoculated to 5 ml of GYP medium (where the glucose was replaced with galactose). Finally, the localization of LpSirA in the LpSirA-venus high expresser strain was examined by confocal microscopy. The result indicated that LpSirA-Venus protein was localized with a regular pattern, such as a spiral in the cytoplasm. In *Bacillus subtilis*, FtsZ in the Z-ring was shown to be localized to the division plates during cell division. In the cellular pole, there are RacA protein which promotes the movement to the cell pole of the nucleoid, and the cell division protein DivIVA which is connecting the RacA to cellular poles. Further, the actin homolog MreB protein and the MinC, D, E and J proteins were localized as a spiral in the cytoplasm. Thus, the author presumed that LpSirA is involved in cell division in *L. paracasei* BL23.

In this thesis, the author found that the existence of sirtuin gene in LAB and their deacetylase enzyme activities in three strains of *L. paracasei*. In addition, the author revealed that the target protein of LpSirA in *L. paracasei* BL23 is 30S ribosomal protein S4 which is involved in protein synthesis. Using immunofluorescent staining, LpSirA protein was shown to be localized to the division plates and cellular poles during cell division. Additionally, using the LpSirA-Venus high expresser strain, LpSirA protein was localized with a regular pattern, such as a spiral in the

cytoplasm of living LAB. In conclusion, the author concluded that the function of sirtuin may likely be involved in protein synthesis control and cell division in *L. paracasei*. Thus, the author expects that sirtuin may serve as a novel criteria to select LAB for developing better probiotics to contribute for the promotion of human health.

審査報告概要

サーチュインは，代謝の適正化やストレス応答などにかかわる長寿遺伝子として注目されている。その本体はNAD^+依存性のタンパク質脱アセチル化酵素であり，染色体ヒストンタンパク質に作用して遺伝子発現を制御する。一方原核生物では未知の部分が多い。本研究では先行研究例のない乳酸菌に着目し，プロバイオティクスとして繁用される *Lactobacillus paracasei* より遺伝子をクローニングし，組換えタンパク質の産生と精製を経て，脱アセチル化酵素活性を検出し，LpsirA と命名した。次に同菌種における標的アセチル化タンパク質の一つを精製し，N 末端アミノ酸配列を決め，30S ribosomal protein S4 であることを同定した。さらに，抗体と蛍光タンパク質融合体を用いて，その細胞内局在が，細胞極，細胞分裂面，らせん構造であることを示した。これら新規の発見は，乳酸菌の生存やストレス応答機構の解明に迫る貴重な一歩である。

よって，審査員一同は博士（生物産業学）の学位を授与する価値があると判断した。