Identification of sirtuin and its target as the ribosomal protein S4 in Lactobacillus paracasei

(Received October 28, 2015; Accepted December 22, 2015)

Hotaka Atarashi,† Shinji Kawasaki,‡ Yoichi Niimura,‡ Naoto Tanaka,§ Sanae Okada,§ Yuh Shiwa,¶ Akihito Endo,† and Junichi Nakagawa†,*

† Department of Food and Cosmetic Science, Graduate School of Bioindustry, Tokyo University of Agriculture, Hokkaido 099-2493, Japan
‡ Department of Bioscience, Tokyo University of Agriculture, Tokyo 156-8502, Japan
§ Culture Collection Center, NODAI Research Institute, Tokyo University of Agriculture, Tokyo 156-8502, Japan
¶ Genome Research Center, NODAI Research Institute, Tokyo University of Agriculture, Tokyo 156-8502, Japan
* Division of Biobank and Data management, Iwate Tohoku Medical Megabank Organization, Iwate Medical University, Iwate 028-3694, Japan

Sirtuin is a protein with an enzymatic activity of NAD+-dependent protein deacetylation. It was first identified in yeast and its homologous genes have been widely found in various organisms. In bacteria, sirtuin gene was first described as cobB, encoding a cobalamin processing enzyme; and later its potential involvement in regulating acetylation levels of metabolic enzymes, transcription factors, chemotactic proteins and others have been reported. In order to study its physiological relevance in probiotic lactic acid bacteria, we analyzed the whole genome of three L. paracasei strains. All strains tested had sirtuin homolog genes designated hereby as sirA, and one of them had an additional gene designated as sirB. Following confirmation of their coding sequences by individual gene cloning, corresponding recombinant proteins have been generated and purified. The enzymatic characterization revealed that the intrinsic NAD+-dependent deacetylation activity of LpSirA (protein encoded by sirA) is comparable to human SIRT1. Furthermore, by blocking sirtuin activity using nicotinamide in vivo, together with an in vitro deacetylation reaction using recombinant LpSirA, we identified one of the target proteins in the lactic acid bacteria as the 30S ribosomal protein S4 (rpsD product).

Key Words: acetylated protein substrate; bacterial sirtuin; Lactobacillus; probiotics; ribosomal protein

Introduction

Sirtuin was originally designated as silent information regulator 2 (SIR2) in yeast, which stands for the silence regulation of mating type genes (Fritze et al., 1997). The gene began to draw wider attention when it showed a positive function in life span control (Anderson et al., 2003; Guarente and Kenyon, 2000; Lin et al., 2000). The similar effect on life span elongation has been demonstrated with nematode, fruit fly, and mammalian cells by molecular approaches (Cohen et al., 2004). After the research was extended to a mammalian system, it has been shown to be involved in the regulation of metabolism, circadian rhythm and genetic stability, and the key downstream control point was unraveled to be TOR-mediated signal transduction (Blagosklonny, 2010; Guarente and Kenyon, 2000).

Evolutional function of sirtuin was postulated to play a role in survival upon starvation, partly because its expression was shown to be up-regulated upon calorie restriction (Cohen et al., 2004). The gene sequences of bacterial sirtuin-homologs have been accumulated in the GenBank, providing a long list of bacterial sirtuins in many archaea-
and eu-bacteria (Frye, 2000). As for the function of these putative ancestral sirtuin homologs, only a few cases have been reported. In *Escherichia coli* and in *Salmonella enterica*, it has been shown to deacetylate acetyl CoA synthetase, thereby activating the enzyme (Starai et al., 2002). While in *Bacillus subtilis*, two deacetylases either requiring or not-requiring NAD⁺ are involved in the deacetylation of the enzyme (Gardner and Escalante-Semerena, 2009; Gardner et al., 2006). In *E. coli*, the chemotactic gene *cheY* product was reported to be deacetylated by CobB protein (Li et al., 2010). It is not clear whether bacterial sirtuin has a similar function in the chromatin remodeling system in eukaryotes. However, as an example of an individual transcription factor, it was reported that, when the acetylated Lysine 180 of RcsB protein (two-component response regulator) was deacetylated by CobB protein, the DNA binding activity of RcsB is increased to activate the transcription of the target gene, *flhDC* in *E. coli* (Thao et al., 2010). In addition, the importance of protein acetylation as a posttranslational modification has been documented (Bernal et al., 2014).

**Materials and Methods**

**Chemicals and strains.** Chemicals were of special grade unless otherwise mentioned. *Lactobacillus paracasei* strains NRIC 0644, NRIC 1917 and NRIC 1981 were obtained from the NRIC culture collection (Tokyo University of Agriculture, Japan). Strains NRIC 1917 and NRIC 1981 were isolated from compost and sugar cane wine, respectively, whereas the origin of NRIC 0644 was unknown (Shiwa et al., 2015). Polyclonal rabbit anti-sera were raised against purified recombinant LpSirA (this study), and were affinity-purified by Japan Lamb Ltd. (Hiroshima, Japan). A rabbit anti-acetylated lysine primary antibody and a donkey anti-rabbit IgG secondary antibody were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA), and GE Healthcare Ltd. (Buckinghamshire, England), respectively. The hydrophobic resin, Butyl-Toyopearl was from TOSHO Corp. (Tokyo, Japan), and DEAE cellulose resin, DE52, was from GE Healthcare (Buckinghamshire, England).
Culture and growth. *L. paracasei* strains were cultured in Lactobacilli MR5 medium (Beckton Dickinson, MD, USA) and *E. coli* was cultured in L broth unless otherwise indicated.

*In silico gene analysis.* The details of the methodology and the basic data of the genome analysis of the strains used in this report have been published elsewhere (Shiwa et al., 2015). In this study, identification of sirtuin genes in the draft genomes was performed by using the data analysis program, *In Silico Molecular Cloning (In Silico Biotechnology, Yokohama, Japan).* For the sequence comparison at the level of the amino acid sequence, the sequences obtained by determining individual cloned DNAs were used as described in the following section using the application program—Keyword Search—of the *In Silico Molecular Cloning program.*

The amino acid sequence similarity values with other organisms were calculated using LALIGN program (EMBL-European Bioinformatics Institute, Hinxton, Cambridge, UK, http://www.ebi.ac.uk/Tools/psa/lalign/).

Cloning of DNAs encoding sirtuin homolog. The three strains of *L. paracasei* were cultured in MR5 broth at 37°C overnight. Genomic DNA was extracted from the cultures as described in DNeasy Blood & Tissue Kit (QIAGEN, Venlo, The Netherlands). From the draft sequences of the genomic DNA of *L. paracasei* strains, primers (sirA-N and sirA-C, Table 1) were designed for the amplification of the sirtuin homologous gene. The amplified products were sub-cloned into pT7 Blue vector by TA cloning (Novagen Merck Millipore, Darmstadt, Germany) and transformed into *E. coli* DH5α (Nippon Gene Co., Ltd., Tokyo, Japan). DNA sequence of the cloned DNA was determined by analyzing dye-labeled extension products using the BigDye® terminator v1.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA, USA) subjected to a DNA sequencer 3100 Avant Genetic Analyzer (Applied Biosystems, Waltham, MA, USA). The sequence data were determined following the sequencing of both directions of the DNAs. The sequencing and cloning primers are listed in the Table 1.

Expression and purification of the recombinant Sirtuin proteins. The cloned individual DNA fragments in the pT7 Blue plasmids were further amplified using cloning primers containing artificially added restriction enzyme cutting sites for subsequent sub-cloning. Underlines in the cloning primer sequences indicate artificially added restriction enzyme recognition sites for subsequent sub-cloning (Table 1). The resulting DNA was sub-cloned into bacterial protein expression vector pET-15b (Novagen Merck Millipore, Darmstadt, Germany) at the cloning site between Bpu11021 and XhoI. The proteins were expressed using *E. coli* BL21 (DE3) strain (Novagen Merck Millipore, Darmstadt, Germany). The DNA sequence was confirmed as described above. The recombinant *LpSirB* was produced as described above; therefore, for the purification, the protein was dissolved in the presence of 8 M urea and subsequently purified as above in the presence of 8 M urea. The urea was removed during dialysis using Sir2 buffer.

Enzymatic kinetics. Kinetic studies were performed using Fluor de Lys® fluorimetric activity measurement kit (Biomial/ENZO, Exeter UK) according to the manufacturer’s instruction. 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 reaction followed Michaelis Menten Kinetics, displaying Km (constant) and Vmax (maximum reaction velocity) of SIRT1 and *LpSirA* on the acetylated substrate. The apparent Km and Vmax of SIRT1 and LpSirA from NRIC 0644, 1917, and 1981, were calculated from a Lineweaver Burk plot obtained in the presence of 3 mM NAD+ (fixed) and 0.2 μg of each enzyme protein in the reaction mixtures. The relative fluorescence unit was measured using a NanoDrop 3300 Fluorospectrometer (Thermo Fisher Scientific, Waltham, MA, USA) and the conversion of the relative fluorescence units to molar units for substrate acetylated peptide was calculated from the standard curve using deacetylated fluorescent peptide substrate (Fluor de Lys® deacetylated standard) provided in the kit.

Screening of the target acetylated proteins and amino acid sequencing. Hereafter, instead of the three strains, a widely studied strain, *L. paracasei* BL23 was used. The putative target proteins of sirtuin in BL23 were first screened by inhibiting sirtuin deacetylase using NAM in the culture medium. Following two successive pre-cultures to ensure logarithmic growth, BL23 was cultured in MRS medium supplemented with 0, 5, 10 or 50 mM NAM for 12 hours and collected by centrifugation at 6,000 rpm. The cells were washed once with buffer A (50 mM Tris-HCl, pH 7.6, supplemented with 50 mM NaCl) and re-suspended in buffer A supplemented with 1 mM dithiothreitol and
Fig. 1. Deduced amino acid sequences of L. paracasei sirtuin aligned with sirtuin of other organisms. Single-headed arrows indicate conserved active center histidines. Double-headed arrows indicate NAD-binding domains (Rossmann fold). The two counterpart proteins of L. rhamnosus are labeled as SirA* or SirB* to indicate their equivalence with LpSirA and LpSirB.

Accession numbers:
- L. paracasei NRIC 0644 GenBank nucleotide ID, AB728561;
- L. paracasei NRIC 1917 GenBank nucleotide ID, AB728562;
- L. paracasei NRIC 1981 GenBank nucleotide ID, AB728563 and AB728564 (SirLp1 and SirLp2, respectively);
- L. paracasei BL23 NCBI Gene ID, 6407162;
- L. rhamnosus GG NCBI Gene ID, 8424228 and 8421081 (SirLp1 homolog and SirLp2 homolog, respectively);
- Bacillus subtilis NCBI Gene ID, 936271;
- Escherichia coli K12 NCBI Gene ID, 945687;
- Salmonella Typhimurium LT2 NCBI Gene ID, 1252739;
- Bifidobacterium longum subsp. infantis ATCC 15697 NCBI Gene ID, 7054471;
- Saccharomyces cerevisiae ATCC 204508 NCBI Gene ID, 851520;
- Homo sapiens NCBI Gene ID, 23411.

0.1 mM PMSF. The cell extracts were then made using a bead crusher (TAITEC Corp., Saitama, Japan) with glass beads (0.1 mm in diameter). At the same time, a cell extract (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. The protein concentration was measured by
Western blot analysis. 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 a PVDF cellulose membrane (Amersham Hybond-P, GE Healthcare Buckinghamshire, UK) for 60 min at 40 V using an electroblotter (Semi-dry type NA-1512, Nihon Eido, Co. Ltd, Tokyo, Japan). Western blotting was done using an anti-sirtuin primary antibody or an acetylated-lysine primary antibody, and a donkey anti-rabbit IgG secondary antibody. For the detection, an enhanced chemiluminescence (ECL) system (Pierce, USA) was used with an Amersham™ ECL™ Prime Western Blotting Detection Reagent (GE Healthcare UK Ltd. Buckinghamshire HP7 9NA, England) and a Light-Capture II (AE-6981, ATTO, Tokyo, Japan).

Purification of target protein from L. paracasei BL23 and western blot analysis. Cells of L. paracasei BL23 strain were cultured in MRS medium supplemented with 50 mM NAM for 12 hours and collected by centrifugation at 6,000 rpm. The cells were washed once using the above-mentioned buffer A, and re-suspended in buffer A supplemented with 1 mM dithiothreitol, 0.1 mM PMSF. The cell extracts were then made using an ultrasonic disintegrator. To purify the candidate proteins in the cell extracts, most of the proteins were first collected by ammonium sulfate precipitation (0–80%), then dialyzed and supplemented again with ammonium sulfate to 25% saturation. The solution was then subjected to a Butyl-Toyopearl column. Proteins were eluted by stepwise decreases in ammonium sulfate concentration and were subjected to SDS-PAGE followed by western blotting to identify the acetylated protein. The fraction eluted with 10% ammonium sulfate containing the targets was then subjected to a DE52 column, and the proteins were eluted by a stepwise increase in NaCl concentration. The elution at 0.5 M NaCl showed a single band of an acetylated 28 kDa protein. The acetylation and deacetylation by LpSirA were confirmed by western blotting using anti-acetyl lysine antibody as described above. The fraction eluted with 10% ammonium sulfate was further concentrated by acetone precipitation (80%). The precipitated protein was resolved in buffer A, and subjected to SDS-PAGE followed by western blotting using an electroblotter (Semi-dry type NA-1512, Nihon Eido, Co. Ltd, Tokyo, Japan).

Cloning of individual DNAs coding for sirtuin homolog from strains NRIC 0644, 1917 and 1981

The sirtuin homolog genes were directly cloned from DNA isolated from the three strains by a PCR cloning method, and the sequences of the cloned DNA were determined by sequencing both directions as described in Section “Materials and Methods”. The DNA sequences obtained were used to determine the corresponding deduced amino acid sequence which was then aligned using the BioEdit Sequence Alignment Editor, as shown in Fig. 1. Based on the deduced amino acid sequences, strains NRIC 0644 and NRIC 1917 were shown to have an ORF 99% homologous to that of strain L. paracasei BL23 (LCABL_27750, NCBI-Gene ID: 6407162), while strain NRIC 1981 had an ORF 99% homologous to that of strain L. paracasei BL23, NRIC 1981 LpSirA, and L. paracasei NRIC 1981 LpSirB.
strains were similar to each other. Two active histidine residue sites, as reported in human SIRT1 (Sanders et al., 2010), were conserved in the clones of all three strains (his327 and his363 of SIRT1, which corresponded to his79 and his113 of LpSirA protein as indicated by single-headed arrows in Fig. 1). The NAD-binding motif, called the Rossmann fold sequence, was also present in all three clones as marked by double-headed arrows in Fig. 1). The NAD-binding domain, called the 28 kDa protein. The solid triangles indicate the position of the 28 kDa protein, and the small arrows indicate the position of NAM supplemented in the medium (indicated at the bottom). The concentration of NAM or NAD+ was 10 mM each. Ten µg of purified recombinant LpSirA was supplemented in the far-right sample. Each sample was incubated at 37°C for 1 hour. The solid triangles indicate the position of the 28 kDa protein, and the small arrows indicate the positions of 27, 29, 31, 40 and 48 kDa, whose acetylation levels were decreased by treatment with LpSirA and NAD+.

Expression and purification of recombinant L. paracasei sirtuin homolog proteins

The cDNA of sirtuin homolog was introduced in the bacterial expression system using E. coli, and the histagged recombinant proteins were affinity-purified. As shown in Fig. 2, recombinant protein preparations appeared homogeneous after SDS-PAGE and Coomassie brilliant blue staining, displaying apparent molecular sizes of 29 kDa for LpSirA and 34 kDa for LpSirB product.

Enzyme activity of the recombinant LpSirA proteins from L. paracasei strains

Comparative studies were performed to examine the deacetylating enzymatic activity of LpSirA proteins derived from the three strains of L. paracasei and human SIRT1 protein. Kinetic parameters were measured to obtain Km and Vmax values as described in Section “Materials and Methods”. The apparent Km values in terms of the acetylated peptide substrate 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 initially calculated from the measurement of the relative fluorescence unit, then converted to molar units according to the standard curve made by deacetylated fluorescent peptide provided in the Fluor de Lys® fluorimetric activity measurement kit. 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, respectively. The enzyme characteristics of the L. paracasei sirtuin proteins were comparable to human SIRT1. As for the optimal temperature for the enzymatic reaction, LpSirA proteins displayed a higher optimal temperature (45–50°C) than SIRT1 (37°C). As for LpSirB, enzymatic activity was not measured, as this protein turned out to be precipitated again after dialysis following affinity column purification.

Identification of the sirtuin-target acetylated proteins in L. paracasei

In an attempt to search for the endogenous substrate acetylated proteins of sirtuin in L. paracasei, the cells were treated with a sirtuin inhibitor, NAM, supplemented in the culture medium. After cell disruption by ultrasonic treatment, the cell extracts were subjected to SDS-PAGE followed by electro-blotting to a cellulose membrane. The results shown in Fig. 3A indicated that the acetylation levels of the 28 kDa protein (indicated by a solid triangle) were significantly elevated with increasing concentration of NAM supplemented in the medium (indicated at the bottom of Fig. 3A). On the other hand, when in vitro deacetylation was performed with purified LpSirA protein in the presence of NAD+ for the cell extracts prepared after culturing L. paracasei cells in the presence of 50 mM NAM, about 6 acetylated proteins with molecular weights of 27, 28, 29, 31, 40 and 48 kDa showed a decrease in signal intensity on western blots (Fig. 3B, indi-
Fig. 4. Purification of the target protein from *L. paracasei* BL23. Coomassie brilliant blue staining (A) and western blot with anti-acetylated lysine antibody (B). Lanes 1: Crude extract (100 µg protein), 2: Ammonium sulfate precipitate (100 µg protein), 3: Fraction after Butyl Toyopearl column (50 µg protein), and 4: Fraction after DEAE-cellulose column (1 µg protein). (C) *In vitro* deacetylation of the purified 28 kDa protein by LpSirA. Lanes 1: Purified 28 kDa protein (0.6 µg without treatment), and 2: Purified 28 kDa protein (0.6 µg the 28 kDa protein was incubated with 5 µg of LpSirA in the presence of 10 mM NAD⁺ for 1 hour at 37°C). The solid triangles indicate the position of the 28 kDa protein.

cated by small arrows for the 27, 29, 31, 40 and 48 kDa proteins, and a solid triangle for the 28 kDa protein). Such a decrease was not observed in the absence of the supplemented recombinant LpSirA, or when the endogenous LpSirA contained in the extract was inhibited in the presence of NAM. Among these deacetylated proteins, the 28 kDa protein was the most significantly deacetylated *in vitro* by LpSirA, and its acetylation levels was increased *in vivo* with NAM. Taken together, the 28 kDa band was identified as the endogenous target sirtuin substrate, which was then subjected to purification. Proteins in the cell extracts were partially purified by ammonium sulfate precipitation, and further purified with two-step column chromatographies using butyl sepharose and DEAE sepharose. The resulting purified acetylated 28 kDa protein (Figs. 4A-4 and B-4) was deacetylated by supplemented LpSirA as shown in Fig. 4C (compare lane 1 vs. 2). The N-terminal amino acid sequence of the 28 kDa target protein was determined by the Edman degradation method to be SRYTGPRWKQ, which was exactly identified to be the 30S ribosomal protein S4 of *L. paracasei* in the data bank.

**Discussion**

In order to elucidate the function of sirtuin in LAB, we generated recombinant sirtuins of LAB and examined the enzymatic kinetics. The recombinant LpSirA proteins displayed deacetylase activity to the acetylated peptide substrate *in vitro*, and the displayed Km values were almost equivalent compared with that of SIRT1, despite the fact that the substrate used in this assay was a human p53-derived acetylated peptide (Avalos et al., 2002), and not a bacterial peptide, implying evolutional retention of the conserved microstructure of the active center. This is also supported by the presence of conserved histidine residues at the corresponding position in the polypeptide sequence and the Rossmann fold commonly recognized as the NAD-binding site.

The deduced amino acid sequence of LpSirA was highly conserved, not only with the previously reported sequences of *L. paracasei* strains Zhang or BL23 deposited in the database, but also with sirtuin from other species, especially at the active site, and the amino acid residues in the NAD-binding-Rossmann fold, indicating its essential role throughout evolution (Sanders et al., 2010). As indicated from the evolutional conservation, the sirtuin gene should play an advantageous role in the *Lactobacillus* species, whether this is essential or not. One such role may be improving the survival rate under stress by regulating the protein synthesis rate, as the target acetylated protein was revealed to be 30S ribosomal protein S4. The function of 30S ribosomal protein S4 was first indicated for mRNA binding as a translational repressor (Tang and Draper, 1990). More recently, it was postulated to function in multiple stages of ribosome assembly by interacting with 16S rRNA (Mayerle and Woodson, 2013). The crystal structure of 30S ribosomal protein S4 was revealed to suggest its function as RNA-binding through a positively-charged domain (Davies et al., 1998). It would be in line with this report that the deacetylation of the 30S ribosomal protein S4 may weaken the RNA-binding to render either alleviation of translational repression or the efficiency of the ribosome assembly, which may slow down growth rate, possibly in response to environmental stress. Also, consistent with this, it is noteworthy that the ribosomal large subunit component MRP10 was found to be the target of eukaryotic mitochondrial sirtuin in humans, and it was shown, using a mouse model system, to decrease the protein synthesis rate, possibly functioning in survival under starving condition (Yang et al., 2010). The acetylation of 30S ribosomal protein S5 in bacteria has been reported, but, to our knowledge, the acetylation of the 30S ribosomal protein S4 has not been reported. Therefore, this is the first evidence that the 30S ribosomal pro-
tein S4 is acetylated and can be deacetylated by LpSirA, at least in Lactobacilli. AbouElfetouh et al. (2015) have reported an extensive global analysis using cobB deletion mutant of E. coli and found S1 acetylated proteins in the bacterium. These proteins were sensitive to CobB and were involved primarily in translation, central metabolism and DNA-centered processes.

There might be further putative endogenous substrates in the LAB yet to be discovered. One such candidate is the transcription factor RcsB, which was reported to be deacetylated by CobB in E. coli and S. enterica. In this case, deacetylation of the RcsB protein was reported to lead to augmentation of its DNA-binding activity (Thao et al., 2010).

Ma and Wood (2011) reported that protein deacetylation by CobB leads to a decreased resistance of E. coli against oxidative stress. Previously, we demonstrated that SIR3 and SIR4 deficiency in yeast resulted in an improved resistance to ethanol and hydrogen peroxide (Matsuda et al., 2011). Lactic acid bacteria are beneficial for the improvement and maintenance of a healthy balance of intestinal bacterial flora. In order to elicit such effects as probiotics, the bacteria need to survive gastric fluid and bile acid in the digestive canal of the host, and preferably to settle on appropriate sites. Regarding the application of LAB as a probiotic, it would be intriguing to find out which target proteins of LAB play a role in the survival against stress in host intestines (van de Guchte et al., 2002).

The results of this study may contribute to the characterization of sirtuin activities in lactic acid bacteria by providing novel criteria for using sirtuin activity for the selection of LAB and Bifidobacterium as probiotics.

Acknowledgments

We are grateful to Ms. Yoko Obuchi, Mrs Yuuya Yoshida, Ryoji Tanaka and Mitsuki Kussakari for technical assistance in the initial part of this study. This work was supported in part by the Grant-in-Aid for Advanced Research Activities in the Graduate School of Tokyo University of Agriculture (TUA), and by Kagome Co. Ltd. HA is a recipient of a Grant-in-Aid for selected Doctoral students in the TUA. We are grateful to Ms. Glaezel Torres MSc. for help in editing the English. We declare that we have no conflict of interest concerning the study described in this paper.

References


