Physiological function analysis of *Lactobacillus plantarum* Y44 based on genotypic and phenotypic characteristics

Yuan Gao,^{1,2} Yujun Liu,¹ Mengying Sun,^{1,2} Heping Zhang,^{1,3} Guangqing Mu,^{1,2*} and Yanfeng Tuo^{1,2*}

¹School of Food Science and Technology, Dalian Polytechnic University, Dalian 116034, China

²Dalian Probiotics Function Research Key Laboratory, Dalian Polytechnic University, Dalian 116034, China

³Key Laboratory of Dairy Biotechnology and Engineering, Ministry of Education, Inner Mongolia Agricultural University, Hohhot 010018, China

ABSTRACT

In our previous studies, *Lactobacillus plantarum* Y44 showed antioxidant activity and favorable gastric and intestinal transit tolerance. In the current study, we investigated the physiological function of *L. plantarum* Y44 based on an analysis of its genotype and phenotype. The complete genome of *L. plantarum* Y44 contained a single circular chromosome of 3,255,555 bp, with a GC content of 44.6%, and a single circular plasmid of 51,167 bp, with a GC content of 38.8%. The *L. plantarum* Y44 genome contained 3,293 genes including 3,112 protein coding sequences, 16 rRNAs, 66 tRNAs, 4 small (s)RNAs, and 95 pseudo genes. *Lactobacillus plantarum* Y44 could metabolize 24 different carbohydrate sources. Nineteen complete phosphoenolpyruvate-dependent sugar phosphotransferase system complex genes and intact Embden-Meyerhof-Parnas pathway and hexose monophosphate pathway enzyme genes, as well as abundant carbohydrate active enzyme genes, were identified in the *L. plantarum* Y44 genome. We also identified genes related to the biosynthesis of exopolysaccharide and surface proteins. Surface proteins played an important role in the *L. plantarum* Y44 adhesion to HT-29 cell monolayers, as evidenced by the removal of cell surface proteins leading to decreased adhesion capacity. The *L. plantarum* Y44 genome contained genes encoding chaperones, intracellular proteases, and 2-component systems, which were associated with the general stress response. Genes encoding bile salt hydrolase, F₀F₁-ATPase, Na⁺/H⁺-antiporter, H⁺/Cl[−] exchange transporter, cyclopropane-fatty acyl-phospholipid synthase, and alkaline shock protein were identified in the *L. plantarum* Y44 genome, which might explain the

strain's favorable gastric and intestinal transit tolerance. Some genes associated with encoding the NADH system, glutathione system, and thioredoxin system were predicted via *in silico* analysis and might account for the strain's ability to scavenge reactive oxygen species. *Lactobacillus plantarum* Y44 was susceptible to 7 antibiotics and did not produce biogenic amines, likely due to the absence of acquired antibiotic resistance genes and amino acid decarboxylase genes. The phenotype profile of *L. plantarum* Y44 was associated with its genetic characteristics, indicating that strains with certain physiological functions can be screened by analyzing their phenotypic and genotypic characteristics. *Lactobacillus plantarum* Y44 has the potential to be used as a starter culture in fermented dairy products.

Key words: *Lactobacillus plantarum*, probiotics, complete genome sequence, phenotype, genotype

INTRODUCTION

Lactobacillus plantarum, which is used to produce dairy products such as cheese, kefir, and yogurt (Todorov and Franco, 2010), is an extremely versatile species that offers various beneficial properties to humans (Seddik et al., 2017). It has the qualified presumption of safety status from the European Food Safety Authority (EFSA) and a generally recognized as safe status from the US Food and Drug Administration. In our previous study, *L. plantarum* Y44 exhibited favorable gastric and intestinal transit tolerance, as well as the ability to scavenge reactive oxygen species (ROS) and protect human colon cancer cell lines Caco-2 and HT-29 against ROS injury (Mu et al., 2018, 2019).

The development of high-throughput genomic approaches has led to a deep understanding of the beneficial properties and applications of lactobacilli. The next-generation sequencing enables a better understanding of physiological properties and applications of *Lactobacillus* strains, including their genes, safety, metabolic properties, and ecological roles (Satokari,

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*Corresponding authors: gq6552005@aliyun.com and tyfqj@aliyun.com

2019; Suez et al., 2020). The EFSA has developed guidance (EFSA FEEDAP Panel et al., 2018) for using a microorganism's genome to unequivocally characterize its antibiotic sensitivity, production of toxic metabolites (i.e., biogenic amines), toxicity, and pathogenicity. Rodrigo-Torres et al. (2019) analyzed safety and biotechnological potential of different *L. plantarum* strains through complete genome comparison. *Lactobacillus rhamnosus* GR-1 was genetically and experimentally predicted to be adapted to the vaginal environment (Petrova et al., 2018). Fei et al. (2018) confirmed the ability of *Lactobacillus amylolyticus* L6 to use raffinose and stachyose by complete genome sequencing and phenotype analysis. Using genomic comparison and corresponding phenotype analysis, Fontana et al. (2019) found that *Lactobacillus helveticus* was adapted to dairy niches, as evidenced by its containing maltose-degrading enzymes and multiple copies of 6-phospho- β -glucosidase genes.

Lactobacillus plantarum Y44 has shown favorable stress tolerance and potential applications in commercial dairy products. Before such commercial applications are undertaken, however, the genotypic and phenotypic characteristics of *L. plantarum* Y44 should be clarified. In this study, we sequenced the complete genome of *L. plantarum* Y44, using the PacBio RS II platform, and performed gene predication and annotation *in silico*. We also determined phenotypic characteristics, including the carbohydrate utilization profile, bile salt hydrolase (BSH) activity, adhesion capacity to HT-29 cells, antioxidant enzyme activity, antibiotic susceptibility, and the production of biogenic amines. Finally, we analyzed the association between the phenotypic characteristics and the genome of *L. plantarum* Y44.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Lactobacillus strains tested in this study were stored in de Man, Rogosa, and Sharpe (MRS) broth medium (Land Bridge Technology, Beijing, China) containing 25% glycerol at -80°C in Dalian Probiotics Function Research Key Laboratory. *Lactobacillus plantarum* Y44, *L. plantarum* ATCC 14917, and *L. rhamnosus* GG (ATCC 53103) were cultured in MRS broth (Land Bridge Technology) at 37°C for 18 h twice before use.

Genomic DNA Isolation and Single Molecule, Real-Time Sequencing

Genomic DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI)

according to the manufacturer's instructions. The concentration and purity of genomic DNA were measured by a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA) and electrophoresis on a 0.7% agarose gel. Sequencing libraries with an average insert size of 20 kb were constructed, and the genomic DNA was sequenced on a PacBio RS II platform (Pacific Biosciences, Menlo Park, CA) with P6-C4 chemistry (Eid et al., 2009). The raw reads were filtered and de novo assembled by the hierarchical genome-assembly process (Chin et al., 2013).

Bioinformatic Analysis

The complete genome of *L. plantarum* Y44 was annotated by National Center for Biotechnology Information (NCBI) Prokaryotic Genomes Automatic Annotation Pipeline (PGAAP; Tatusova et al., 2016). A circular genome map of *L. plantarum* Y44 was visualized by CGview (Stothard and Wishart, 2005). Average nucleotide identity values of *L. plantarum* Y44 compared with other *L. plantarum* strains were calculated using JSpeciesWS (Richter et al., 2016). Carbohydrate active enzyme genes (CAZymes) were searched against the CAZy database (Lombard et al., 2014). The metabolic pathways of *L. plantarum* Y44 were predicted by KofamKOALA to search against the KEGG database (Aramaki et al., 2019). The presence of antibiotic resistance genes was detected using the Resfams and CARD database (Gibson et al., 2015; Jia et al., 2017b). ResFinder was used for detecting acquired antibiotic resistance genes (Zankari et al., 2012). The sequences and annotations of other strains analyzed in this study were obtained from the NCBI database (<http://www.ncbi.nlm.nih.gov>).

Carbohydrate Utilization Profile

The carbohydrate utilization profile of *L. plantarum* Y44 was tested with an API 50 CHL kit (bioMérieux SA, Marcy-l'Étoile, France) and 49 selected carbohydrate sources following the manufacturer's instructions, with *L. plantarum* ATCC 14917 as the control.

BSH Activity

The BSH activity of *L. plantarum* Y44 was assessed as described by Beck et al. (2019). *Lactobacillus plantarum* Y44 was streaked on MRS agar containing 0.5% taurodeoxycholic acid (wt/vol) (TDCA, Aladdin, Shanghai, China) or 0.5% glycodeoxycholic acid (GDCA, Aladdin) and anaerobically incubated at 37°C for 48 h. Deconjugation of TDCA or GDCA resulted

in a white precipitate of deoxycholate in the vicinity of bacterial colonies with BSH activity. *Lactobacillus rhamnosus* GG was used as a positive control.

Quantitative determination of the BSH activity was done by measuring the liberation of amino acids (glycine or taurine) from TDCA or GDCA, as described by Fontana et al. (2019). Bile salt hydrolase activity was expressed in units per milliliter because 1 unit of BSH activity was defined as the amount of enzyme that liberated 1 mmol of glycine (TDCA as substrate) or taurine (GDCA as substrate) per minute.

Capacity for Adhesion to HT-29 Cells

Human colon cancer cells (line HT-29, Cell Bank of the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China) were routinely cultured at 37°C in a 5% CO₂ atmosphere in RPMI-1640 medium (Gibco Life Technologies, Grand Island, NY) supplemented with 10% (vol/vol) fetal bovine serum (Gibco Life Technologies), 100 U/mL penicillin, and 100 mg/mL streptomycin (Meilun Biotechnology, Dalian, China).

The adhesion capacity of *L. plantarum* Y44 to HT-29 cells was investigated according to the method described by Lee et al. (2016b), with some modifications. Briefly, HT-29 cells were seeded at a density of 2×10^5 cells/well on a 12-well microplate (Corning Inc., Corning, NY) in 1 mL of RPMI-1640 medium at 37°C for 48 h to form cell monolayers. *Lactobacillus plantarum* Y44 was resuspended in RPMI-1640 medium to a final concentration of 5×10^8 cfu/mL, and 1 mL of the suspension was added to each well of the 12-well microplate. After a 2-h incubation, the cells were washed 5 times with PBS (pH 7.4, Gibco Life Technologies) to remove nonadherent bacteria. HT-29 cells were lysed by the addition of 0.5% (vol/vol) Triton-X100 (Solarbio Life Science, Beijing, China), and adherent viable counts of *L. plantarum* Y44 were determined by a pour plate method using MRS agar after incubation for 48 h at 37°C. The adhesion capacity was defined as the number of adhered bacterial cells per HT-29 cell. *Lactobacillus rhamnosus* GG was used as a positive control.

The adhesion ability of the *L. plantarum* Y44 to HT-29 cells was also evaluated considering the effect of their cell surface proteins. Cell surface proteins of *L. plantarum* Y44 were removed by mixing with LiCl (5 M) and incubating at 37°C for 30 min. The mixture was then centrifuged ($5,000 \times g$, 10 min, 4°C) to remove the supernatant. The viable counts of LiCl-treated *L. plantarum* Y44 were determined by a pour plate method using MRS agar after serial dilution in maximum re-

covery. The bacterial cells were washed twice with PBS and resuspended in RPMI-1640 medium (Gibco Life Technologies) to a final concentration of 5×10^8 cfu/mL for the assay of the ability to adhere to HT-29 cells as described above.

Superoxide Dismutase and Glutathione Peroxidase Activity Assay

The superoxide dismutase (SOD) and glutathione peroxidase (GPx) activities were determined using the detection kit provided by Nanjing Jiancheng Bioengineering Institute (Nanjing, China) according to the manufacturer's instruction.

Antibiotic Susceptibility

The antibiotics ampicillin, vancomycin, gentamicin, kanamycin, erythromycin, clindamycin, tetracycline, and chloramphenicol (Sangon Biotech, Shanghai, China) were chosen for antibiotic susceptibility testing of lactobacilli according to EFSA FEEDAP Panel et al. (2018). Minimum inhibitory concentration was determined using the standard microdilution method for *Lactobacillus* antibiotic susceptibility testing as reported in the ISO10932/IDF233 2010 document (International Dairy Federation, 2010). Briefly, each antibiotic was carefully weighed, dissolved in the appropriate solvent, filter-sterilized (0.22 µm, Merck Millipore, Darmstadt, Germany), and kept at -80°C as a stock antibiotic solution. Specific concentrations of antibiotic working solutions were prepared by serial 2-fold dilution of stock antibiotic solutions, and the concentration range was selected according to the ISO10932/IDF233 2010 document. Overnight *Lactobacillus* cultures were adjusted to approximately 3×10^8 cfu/mL in lymphocyte separation medium (LSM; ISO-Sensitest broth, Oxoid, Basingstoke, UK, supplemented with 10% vol/vol MRS broth). Then, the bacterial suspension was diluted 500 times in LSM and mixed with the antibiotic working solutions. The mixture was incubated at 28°C for 48 h. The MIC was defined as the lowest concentration of an antibiotic that prevented visible growth of a test organism in a broth microdilution method under the conditions specified in the ISO10932/IDF233 2010 document (International Dairy Federation, 2010). The quality control strain *L. plantarum* ATCC 14917 included in that document was used for comparisons and interlaboratory range analysis. Antibiotic susceptibility of *L. plantarum* Y44 was calculated according to microbial cutoff values (µg/mL), as recommended by EFSA FEEDAP Panel et al. (2018).

Production of Biogenic Amines

Decarboxylase medium plate was prepared according to the method described by Bover-Cid and Holzapfel (1999). *Lactobacillus plantarum* Y44 was cultivated 5 times in MRS broth (Land Bridge Technology) supplemented with 0.1% (wt/vol) of each amino acid (tyrosine, histidine, ornithine, lysine, tryptophan, phenylalanine, and arginine, respectively; Solarbio Life Science) and 0.005% pyridoxal-5-phosphate (Solarbio Life Science) for 24 h to induce decarboxylase expression. Then, 10 μ L of the bacterial suspension was inoculated onto the decarboxylase medium plate and incubated for 96 h at 37°C. The appearance of a purple color on the decarboxylase medium plate indicated that the strain could produce biogenic amines (Chang and Chang, 2012). *Lactobacillus rhamnosus* GG was used as the control.

Statistical Analysis

All tests were performed in triplicate. Data are presented as mean $\pm$ standard deviation. One-way ANOVA was performed with SPSS (Version 20.0, SPSS Inc.,

Chicago, IL) to verify significant differences between samples. The results were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

General Genome Features of *L. plantarum* Y44

The complete genome of *L. plantarum* Y44 consisted of a circular chromosome of 3,255,555 bp and a 51,167-bp plasmid, named pY44-1, as shown in Figure 1 and Table 1. A total of 3,194 genes were predicted by PGAAP, including 3,013 protein coding sequences, 16 rRNAs, 66 tRNAs, 4 sRNAs, and 95 pseudo genes. The average nucleotide identity values of different *L. plantarum* strains are shown in Figure 2. *Lactobacillus plantarum* Y44 clustered with *L. plantarum* ssp. *plantarum* ST-III (*L. plantarum* ST-III) and *L. plantarum* CAUH2, indicating that *L. plantarum* Y44 had the closest evolutionary relationship with these strains. The complete genome of *L. plantarum* Y44 has been deposited in the NCBI GenBank database under the accession number CP030244-CP030245.

Figure 1. Circular genome maps of *Lactobacillus plantarum* Y44. For both the chromosome and the plasmid, the first and second circles (starting with the outer circle) illustrate the predicted protein coding sequences (CDS), tRNA, and rRNA on the forward and reverse strands. The third circle represents the G+C content of the genome. The fourth circle represents GC skew $(G - C)/(G + C)$. The fifth circle represents the genome size.

Carbohydrate Metabolism and Related Genes of *L. plantarum* Y44

The carbohydrate utilization profile of *L. plantarum* Y44 was determined with an API 50 CHL kit. As shown in Table 2, *L. plantarum* Y44 could metabolize 24 kinds of carbohydrate sources. *Lactobacillus plantarum* Y44 was not capable of fermenting D-raffinose, even though a α -galactosidase gene (*mela*, DQQ11_14885) was identified in its genome. Lactose represents the principal carbohydrate component in dairy niches. *Streptococcus thermophilus* and *L. bulgaricus* normally metabolize lactose and release galactose into the surrounding environment, resulting in the accumulation of residual galactose and unfermented lactose in yogurt making (Anbukkarasi et al., 2014). As shown in Table 2, *L. plantarum* Y44 strongly fermented lactose and galactose, which would solve the problem of residual galactose and lactose in yogurt fermentation. A total of 3 copies of β -galactosidase genes (DQQ11_14810, DQQ11_14875, DQQ11_14880) were identified in the *L. plantarum* Y44 genome. The Leloir pathway is a crucial lactose metabolism pathway, and genes encoding the complete pathway were identified in the *L. plantarum* Y44 genome, including UDP-glucose-hexose-1-phosphate gene, galactokinase gene, UDP-glucose 4-epimerase gene, and aldose 1-epimerase gene (Supplemental Table S1; <https://doi.org/10.3168/jds.2019-18047>). The redundant β -galactosidase genes, including the Leloir pathway gene, might indicate that *L. plantarum* Y44 possesses better suitability for dairy niches. Beck et al. (2019) found that the carbohydrate utilization profile of different *L. plantarum* strains could vary, as shown through a comparison of carbohydrate fermentation patterns among *L. plantarum* ATG-K2, ATG-K6, and ATG-K8 by API50 CHL kit.

Lactobacillus plantarum is a versatile and flexible species that is able to grow on a wide variety of sugar sources. This phenotypic trait is associated with the genes encoding proteins related to carbohydrate transport and metabolism. The majority of transporters are within the phosphoenolpyruvate-dependent sugar

phosphotransferase system (PTS). The complete PTS encoded by the *L. plantarum* Y44 genome includes general enzyme I gene (EI, DQQ11_05360), phospho-carrier protein HPr gene (DQQ11_05355), 19 complete substrate-specific enzyme II (EII) complexes genes, and genes for several incomplete complexes (Supplemental Table S2; <https://doi.org/10.3168/jds.2019-18047>). In the *L. plantarum* Y44 genome, the genes for glucose-glucoside (Glc), fructose-mannitol (Fru), mannose-fructose-sorbose (Man), and lactose-N,N'-diacetylchitobiose- β -glucoside (Lac) family EII complexes were present as multiple copies, while the genes for galactitol (Gat), L-ascorbate (Asc), and glucitol (Gut) family of EII complexes were present as single copies. In addition to those in the PTS system, *L. plantarum* Y44 possessed dozens of other carbohydrate transporters. The substrate specificities of *L. plantarum* Y44 PTS and other carbohydrate transporters could not be predicted. Kleerebezem et al. (2003) explained that some carbohydrate transporter systems are known to import more than one substrate.

Lactobacillus plantarum is grouped among the facultative heterofermentative lactobacilli, which ferment sugars via the Embden-Meyerhof-Parnas (EMP) pathway or the hexose monophosphate (HMP) pathway, leading to homolactic and heterolactic fermentation profiles, respectively (Kleerebezem et al., 2003). *Lactobacillus plantarum* degraded glucose via the EMP pathway to produce pyruvate, which was subsequently converted into D- and L-lactate by lactate dehydrogenase enzymes (Liu et al., 2015). The genes encoding enzymes involved in the intact EMP and HMP pathways were predicted in the *L. plantarum* Y44 genome (Supplemental Table S1). Two D-lactate dehydrogenase genes (*ldhD*) and 5 L-lactate dehydrogenase genes (*ldhL*, Supplemental Table S1) were found in the *L. plantarum* Y44 genome. Two L-lactate dehydrogenase genes and one D-lactate dehydrogenase gene were identified in the genome of *L. plantarum* 5-2 (Liu et al., 2015). *Lactobacillus plantarum* Y44 displayed redundancy in its lactate dehydrogenase-encoding genes, indicating that the strain has a pyruvate-dissipating capacity in fermenta-

Table 1. Genomic information of *Lactobacillus plantarum* Y44

Item	Complete genome	Chromosome	Plasmids
Size (bp)	3,306,722	3,255,555	51,167
GC content (%)	44.5	44.6	38.8
Gene	3,194	3,142	52
Protein coding sequence	3,103	2,967	46
tRNA	66	66	—
rRNA	16	16	—
Small (s)RNA	4	4	—
Pseudo gene	95	89	6
Methylation site	10	10	—

Figure 2. Average nucleotide identity (ANI) values of *Lactobacillus plantarum* strains. The numbers indicate the ANI of each *L. plantarum* strain.

tion. Aside from the lactate dehydrogenase genes, the *L. plantarum* Y44 genome contains a relatively large number of other pyruvate-dissipating enzymes, which are predicted to catalyze the production of other metabolites, including formate, acetate, ethanol, acetoin, oxaloacetate, and acetyl-CoA (Supplemental Table S1). Formate, acetate, ethanol, acetoin, and oxaloacetate are important flavor compounds of fermented milk or cheese (Marshall, 1987). The *L. plantarum* Y44 genome did not encode proteins related to the intact citrate acid cycle (tricarboxylic acid cycle), but some genes in this pathway were present (Supplemental Table S1). It was previously reported that the genomes of *L. planta-*

rum 5-2 (Liu et al., 2015) and *L. plantarum* ZJ316 (Li et al., 2016) also did not encode an intact TCA cycle.

The analysis of carbohydrate active enzyme genes using the CAZy database showed that the *L. plantarum* Y44 genome contains 203 genes in the 5 CAZymes gene families as follows: 28 auxiliary activity genes, 18 carbohydrate-binding module genes, 30 carbohydrate esterase genes, 75 glycoside hydrolase genes, and 52 glycosyl transferase genes. These numbers of carbohydrate active enzyme genes were relatively larger than those found for *L. plantarum* KLDS1.0391 and *L. plantarum* ZLP001, which harbored 119 genes and 94 genes, respectively (Zhang et al., 2018; Jia et al.,

2017a). However, the carbohydrate utilization capacity of *L. plantarum* KLD1.0391 and *L. plantarum* ZLP001 are unknown.

Genes Related to Proteolysis and Amino Acid Metabolism in *L. plantarum* Y44

Dairy microbes have abundant proteolytic and amino acid metabolism genes that enable uptake of nutrients present in the dairy environment (Stefanovic and McAuliffe, 2018). *Lactobacillus* strains are generally equipped with protein-degradation machinery to create a selective advantage for growth. Unlike other *Lactobacillus* strains, *L. plantarum* did not appear to contain a gene encoding the extracellular protease Prt, which is involved in primary breakdown of proteins (Kleerebezem et al., 2003). As for the *L. plantarum* Y44 genome, it did not encode extracellular protease Prt. The *L. plantarum* genome encoded peptide uptake systems for transporting oligopeptides into the cells as nitrogen sources. Oligopeptide and di-/tripeptide (DtpT) uptake systems are major peptide uptake systems in *L. plantarum* (Kleerebezem et al., 2003). We found that the *L. plantarum* Y44 genome contained intact *opp* peptide uptake system genes, including *opp* operon (*oppA-oppF*), and 7 redundant copies of *oppA* genes (Supplemental Table S3; <https://doi.org/10.3168/jds.2019-18047>). Furthermore, *L. plantarum* Y44 contained a gene encoding the *dtpt* peptide uptake system, which could transport DtpT (Supplemental Table S3). *Lactobacillus plantarum* Y44 contained 19 genes encoding intracellular peptidases, such as aminopeptidase, endopeptidase, proline-specific peptidase, dipeptidase, and tripeptidase (Supplemental Table S3). Milk is relatively poor in free amino acids, and peptidases provide a selective advantage for bacteria growing in this medium (Bolotin et al., 2001).

Amino acid catabolism has an impact on fermentation regulation and on the flavor of dairy products. Similar to other *L. plantarum* strains, the *L. plantarum* Y44 genome encodes complete pathways for the biosynthesis of most amino acids, other than the branched-chain amino acids valine, leucine, and isoleucine. The capability for amino acid biosynthesis supports *L. plantarum* Y44 growth and survival in environments deficient in amino acids. A total of 13 amino acid aminotransferase genes were identified in the genome of *L. plantarum* Y44 (Supplemental Table S3). Amino acids can be converted into their corresponding α -keto acids via catalysis by aminotransferase (Liu et al., 2008). The α -keto acids are subsequently converted into aldehydes, alcohols, and esters, which are important aroma compounds in dairy products.

Table 2. Carbohydrate fermentation patterns of *Lactobacillus plantarum* Y44¹

Sugar	Strain ²	
	Y44	ATCC14917
Control	—	—
Glycerol	—	—
Erythritol	—	—
D-Arabinose	—	—
L-Arabinose	+	+
D-Ribose	+	+
D-Xylose	—	—
L-Xylose	—	—
Adonitol	—	—
Methyl- β D-xylopyranoside	—	—
D-Galactose	+	+
D-Glucose	+	+
D-Fructose	+	+
D-Mannose	+	+
D-Sorbose	—	—
D-Rhamnose	—	—
Dulcitol	—	—
Inositol	—	—
D-Mannitol	+	+
D-Sorbitol	+	+
Methyl- α D-mannopyranoside	+	+
Methyl- α D-glucopyranoside	—	—
N-Acetylglucosamine	+	+
Amygdalin	+	+
Arbutin	+	+
Esculin ferric citrate	+	+
Salicin	+	+
D-Cellobiose	+	+
D-Maltose	+	+
D-Lactose	+	+
D-Melibiose	+	+
D-Sucrose	+	+
D-Trehalose	+	+
Inulin	—	—
D-Melezitose	+	+
D-Raffinose	—	+
Starch	—	—
Glycogen	—	—
Xylitol	—	—
Gentiobiose	+	+
D-Turanose	+	+
D-Lyxose	—	—
D-Tagatose	—	—
D-Fucose	—	—
L-Fucose	—	—
D-Arabitol	—	—
L-Arabitol	—	—
Gluconate	+	+
2-Keto-gluconate	—	—
5-Keto-gluconate	—	—

¹Fermentation results are indicated as follows: +, positive; —, negative.

²Y44 = *L. plantarum* Y44; ATCC14917 = *L. plantarum* ATCC14917.

Capacity for Adhesion to HT-29 Cell Monolayers and Adhesion Factors of *L. plantarum* Y44

Adhesion of *L. plantarum* Y44 to HT-29 cell monolayers is shown in Figure 3, with *L. rhamnosus* GG as a positive control. *Lactobacillus plantarum* Y44 exhibited the same capacity for adhesion to HT-29 cell monolayers.

ers as *L. rhamnosus* GG ($P > 0.05$). When *L. plantarum* Y44 and *L. rhamnosus* GG were treated with LiCl to partially remove cell surface proteins, the cell viability of *L. plantarum* Y44 was decreased to 50%. We then adjusted the concentration of living *L. plantarum* Y44 to 5×10^8 cfu/mL after LiCl treatment to match the concentration of untreated *L. plantarum* Y44. Adhesion of LiCl-treated *L. plantarum* Y44 to HT-29 cells was significantly lower than that of untreated *L. plantarum* Y44 ($P < 0.05$), indicating that surface proteins might account for the adhesion capacity of *L. plantarum* Y44. Previous studies demonstrated that the ability of some *Lactobacillus* strains to adhere to the intestinal mucosa is attributable to their cell surface proteins (Monteagudo-Mera et al., 2019; Meng et al., 2014). These reports are in accordance with our result that the adhesion capacity of *L. plantarum* Y44 was reduced by removing cell surface proteins.

Monteagudo-Mera et al. (2019) inferred that cell surface proteins might work as adhesion mediators when lactobacilli adhere to intestinal epithelial cells. *Lactobacillus plantarum* Y44 contains genes encoding extracellular proteins such as LPxTG-type anchors protein, LysM domains protein, and WxL domain protein (Supplemental Table S4; <https://doi.org/10.3168/jds.2019-18047>). These proteins might play a role in adhesion or binding to other cells or proteins, because they contain mucus-binding, fibronectin-binding, aggregation-promoting, intercellular adhesion, or cell clumping domains that are involved in cell-envelope binding

(Kleerebezem et al., 2003). *Lactobacillus plantarum* Y44 also contained 2 mucus-binding protein genes and a fibronectin/fibrinogen-binding protein gene (Supplemental Table S4). A previous study demonstrated that these 2 kinds of proteins play an important role in adhesion and colonization of intestinal mucosal surfaces or fibronectin (Altermann et al., 2005).

Exopolysaccharide (EPS) is a high molecular weight carbohydrate polymer secreted by many microorganisms, and it generally plays a role in the nonspecific interactions of lactobacilli with epithelial cells (Al-Dhaheri et al., 2017). The EPS-producing cultures used as starters or adjuncts in making yogurt and cheese can positively influence product characteristics such as viscosity and syneresis for yogurt and moisture and firmness for cheese (Che et al., 2019). In our previous study, EPS of *L. plantarum* Y44 was composed of mannose, glucuronic acid, glucosamine, glucose, and galactose, and it exhibited antioxidant activity (Liu et al., 2019). The biosynthesis of EPS involves sugar uptake, nucleotide sugar synthesis, and polysaccharide synthesis and export (Xiong et al., 2019). Predicted pathways of sugar uptake and nucleotide sugar synthesis of *L. plantarum* Y44 are shown in Figure 4A. Various sugar sources are transported intracellularly by corresponding transporters and then metabolized to their corresponding nucleotide sugars, such as GDP-D-mannose, UDP-N-acetyl- α -D-glucosamine, UDP-glucose, UDP-galactose, and UDP-glucuronate, as previously reported (Cui et al., 2017). However, the genotype and phenotype did not completely coincide in our study. For example, the corresponding coding genes for these enzymes, including those responsible for the catalytic formation of GDP-D-mannose from mannose-6-P and the catalytic formation of UDP-glucuronate from UDP-glucose, were not found in the genome of *L. plantarum* Y44 via in silico KEGG pathway analysis. The predicted EPS synthesis gene cluster of *L. plantarum* Y44 is shown in Figure 4B and Supplemental Table S5 (<https://doi.org/10.3168/jds.2019-18047>). Compared with *L. plantarum* WCFS1, *L. plantarum* Y44 did not contain genes encoding the *cps1* cluster. In addition, the *cps2* gene clusters of *Lactobacillus plantarum* strains were diverse; *cps2FGHI* genes of *L. plantarum* Y44 had low homology with those of *L. plantarum* WCFS1, whereas the *cps2FGHI* genes of *L. plantarum* Y44 were highly homologous with the *cps4FGHI* genes of *L. plantarum* WCFS1. Lee et al. (2016a) found that the first 5 genes (*cps2ABCDE*) in the *cps2* gene cluster of *L. plantarum* Lp90 and *L. plantarum* SF2A35B were highly homologous to those of *L. plantarum* WCFS1, and the remaining genes in the *cps2* gene cluster were unique. The *cps3* and *cps4* gene clusters were highly homologous between *L. plantarum* Y44 and *L. plantarum* WCFS1. Jiang and Yang (2018)

Figure 3. The adhesion capacity of *Lactobacillus plantarum* Y44 to HT-29 cells. Y44 = *L. plantarum* Y44; LGG = *Lactobacillus rhamnosus* GG. The results are expressed as means $\pm$ SD ($n = 3$). Different lowercase letters (a and b) on columns indicate a significant difference in the number of adhered bacterial cells per HT-29 cell between different *Lactobacillus* strains at the 0.05 level. The same letter indicates no significant difference at the 0.05 level.

reported that the *cps* gene clusters of 43 *L. plantarum* strains varied considerably mainly because the strains fitted different circumstances.

Genes Encoding Stress-Related Proteins of *L. plantarum* Y44

Lactobacillus strains often encounter adverse conditions during fermentation, such as acid stress and oxi-

dativ stress. In addition, a prerequisite for probiotics is their ability to survive passage through the human gastrointestinal tract, before exerting probiotic effects in the colon (Baccouri et al., 2019). *Lactobacillus plantarum* Y44 demonstrated favorable transit tolerance and oxidative stress tolerance in our previous studies (Mu et al., 2018, 2019). This phenotypic trait is attributable to the high number of genes encoding proteins involved in stress response. The *L. plantarum* Y44

Figure 4. Genes of sugar metabolism for exopolysaccharide (EPS) biosynthesis of *Lactobacillus plantarum* Y44. (A) Pathways of nucleotide sugar synthesis, where locus tags (DQQ11_...) refer to the enzymes involved in the pathways, dotted arrows correspond to enzymatic reactions from inactive pathways, and red lettering represents nucleotide sugars. (B) The EPS gene cluster of *L. plantarum* Y44. Arrows with different colors represent genes belonging to different EPS biosynthesis gene clusters of *L. plantarum* Y44, with red representing genes that belong to the *cps2* cluster, green representing genes that belong to the *cps3* cluster, yellow representing genes that belong to the *cps4* cluster, orange representing other genes related to EPS biosynthesis, and black representing hypothetical protein. PTS = phosphotransferase system; GDP = guanine diphosphate; UDP = uridine diphosphate.

genome contained several genes encoding stress-related proteins, as listed in Supplemental Tables S6 and S7 (<https://doi.org/10.3168/jds.2019-18047>).

Genes encoding chaperones and intracellular proteases were identified in the genome of *L. plantarum* Y44, including *groES-groEL* chaperone operons, *hrcA-grpE-dnaK-dnaJ* chaperone operons, and the protease-encoding genes *hslU*, *hslV*, *lon*, *clpC*, *clpL*, *clpP*, *clpE*, *clpB*, and *clpX* (Supplemental Table S6). Chaperones and proteases are helpful for preventing newly synthesized polypeptide chains and assembled subunits from aggregating and misfolding into nonfunctional structures under a stress condition (Lv et al., 2017). Two-component systems (TCS) act as stimulus-response coupling systems in bacteria (Zschiedrich et al., 2016). At least 14 TCS pairs belonging to 6 families were identified in the genome of *L. plantarum* Y44 (Supplemental Table S7). *Lactococcus lactis* ssp. *cremoris* MG1363 TCS mutants were previously reported to be susceptible to extreme pH, osmotic, or oxidative conditions and to the regulation of phosphatase activity (O'Connell-Motherway et al., 2000). The VicR-VicK system and PhoR-PhoB system were verified to play key roles in regulating stress responses (Deng et al., 2007; Crépin et al., 2011).

In addition to other general stresses, lactic acid-producing bacteria must efficiently deal with the acidification of their local environment (Kleerebezem et al., 2003). As reported in our previous study, the survival rate of *L. plantarum* Y44 in simulated gastric juice for 3 h was 86.01%, indicating that *L. plantarum* Y44 possesses favorable acid tolerance (Mu et al., 2018). The F_0F_1 -ATPase gene, Na^+/H^+ -antiporter gene, and H^+/Cl^- exchange transporter gene were identified in the genome of *L. plantarum* Y44, and they were presumed to serve as major regulators of intracellular pH to allow bacteria to survive in acidic environments (Kleerebezem et al., 2003; Iyer et al., 2002). Similar acid-resistance mechanisms were also found in *L. amylolyticus* L6 by complete genome sequence analysis (Fei et al., 2018).

Additionally, the survival rate of *L. plantarum* Y44 in simulated intestinal fluid for 8 h was 85.53% (Mu et al., 2018). Cyclopropane-fatty acyl-phospholipid (CFP) synthase, alkaline shock protein, and BSH were regarded as key proteins for protecting *Lactobacillus* stains against alkaline or bile stress (Hamon et al., 2011; Koskenniemi et al., 2011). Two copies of alkaline shock protein genes were identified in the *L. plantarum* Y44 genome, and they were expected to play a role in pH tolerance (Koskenniemi et al., 2011). In addition, the gene encoding CFP synthase was present in the genome of *L. plantarum* Y44 (Supplemental Table S6); this enzyme was previously reported to be the major regulator for maintaining the integrity of the cell enve-

lope and homeostasis against bile salt stress (Hamon et al., 2011; Müller et al., 2014).

Bile salt hydrolases are enzymes that hydrolyze the amide bond of conjugated bile salts, liberating the amino acid moiety from the steroid core and generating deconjugated bile salts (Allain et al., 2018). The BSH activity of *L. plantarum* Y44 was determined by qualitative and quantitative methods. As shown in Figure 5A, a white precipitate of deoxycholate in the vicinity of *L. plantarum* Y44 bacterial colonies appeared due to deconjugation of TDCA and GDCA, respectively, indicating that *L. plantarum* Y44 possessed BSH activity. With TDCA and GDCA as substrates, the BSH activities of *L. plantarum* Y44 were 0.81 and 1.25 U/mL, respectively, with no significant difference ($P > 0.05$) compared with those of *L. rhamnosus* GG, as shown in Figure 5B. *Lactobacillus plantarum* Y44 and *L. rhamnosus* GG exhibited the same BSH activities with different substrates. The *L. plantarum* Y44 genome contained 4 *bsh* genes, which were predicted to be highly homologous to the genes of *L. plantarum* WCFS1 and *L. plantarum* ST-III. Four BSH genes were previously identified in the genomes of *L. plantarum* WCFS1 and *L. plantarum* ST-III, with the *bsh1* gene being responsible for the major bile salt hydrolysis activity (Lambert et al., 2008; Ren et al., 2011).

Lactic acid bacteria are anaerobic or facultative anaerobic microorganisms that often encounter oxidative stress during fermentation. Odamaki et al. (2011) found that co-cultivating LAB that had ROS-scavenging ability improved the survival of *Bifidobacterium* in fermented milk. Our previous studies demonstrated that *L. plantarum* Y44 can scavenge extracellular ROS, inhibit the production of intracellular ROS, and activate the Nrf-2/Keap-1/ARE signaling pathway of Caco-2 cells (Mu et al., 2018, 2019). We also found that the SOD and GPx activities of *L. plantarum* Y44 were 16.37 and 17.78 U/mg of protein, respectively. Superoxide dismutase and GPx are key enzymes in the defense against the adverse effects of ROS in living organisms (Wen et al., 2016). We identified genes encoding proteins or enzymes related to scavenging ROS in the *L. plantarum* Y44 genome, including catalase and peroxidase genes (Supplemental Table S6). These enzymes neutralize hydrogen peroxide to nontoxic water (Zhang et al., 2018). Complete NADH system genes, including 5 NADH oxidase genes and 2 NADH peroxidase genes, were identified in the *L. plantarum* Y44 genome (Supplemental Table S6). NADH oxidase cooperating with NADH peroxidase can directly quench hydrogen peroxide and enable ROS degradation (Jänsch et al., 2011; Kang et al., 2013). In addition, we found that the *L. plantarum* Y44 genome contains genes encoding the

Figure 5. Bile salt hydrolase (BSH) activity of *Lactobacillus plantarum* Y44 by (A) de Man, Rogosa, and Sharpe agar plate determination, and (B) quantitative determination. The results are expressed as means $\pm$ SD ($n = 3$). Different lowercase letters (a and b) on columns indicate a significant difference at the 0.05 level in taurodeoxycholic acid (TDCA) substrate; different uppercase letters (A and B) on columns indicate a significant difference at the 0.05 level in the glycodeoxycholic acid (GDCA) substrate. The same letter indicates no significant difference at the 0.05 level.

complete glutathione (GSH) system. The predicted genes encoding the GSH system of *L. plantarum* Y44 included the GSH biosynthesis bifunctional protein gene, GSH peroxidase gene, and GSH reductase gene (Supplemental Table S6). The GSH system detoxifies hydrogen peroxide and lipid peroxyl radicals by regulating the protein dithiol/disulfide balance (Zhang et al., 2018). The thioredoxin system and glutaredoxin catalyze GSH-dependent disulfide reductions to remove ROS at high reaction rates, with glutaredoxin having a thioredoxin-like activity profile (Jordan et al., 1997; Arnér and Holmgren, 2000). The *L. plantarum* Y44 genome harbored the genes encoding the complete thioredoxin system and glutaredoxin, including 5 thioredoxin genes, 1 thioredoxin reductase gene, 1 thiol peroxidase gene, and 1 glutaredoxin gene (Supplemental Table S6). *Lactobacillus plantarum* Y44 possesses SOD activity; however, the SOD encoding gene was not found

in its genome. *Lactobacillus plantarum* compensates for the lack of SOD with a high intracellular accumulation of Mn^{2+} , which can scavenge oxygen radicals (Kleerebezem et al., 2003). We found that the *L. plantarum* Y44 genome contained genes encoding a Mn^{2+} accumulation system, including P-type manganese translocating ATPase gene, *mts* operon (*mtsABC*), and *mntH* family transporter genes (Supplemental Table S6). This Mn^{2+} accumulation system can regulate transportation and accumulation of Mn, as well as strengthen *L. plantarum* tolerance to oxidative stress (Kleerebezem et al., 2003).

Antibiotic Susceptibility and Antibiotic Resistance Genes of *L. plantarum* Y44

Antibiotic susceptibility of *L. plantarum* Y44 was assessed according to the recommendations of the EFSA. *Lactobacillus plantarum* Y44 exhibited antibiotic susceptibility within the recommended cut-off values as shown in Table 3. The results from CARD database searching showed that *L. plantarum* Y44 did not contain strict or perfect antibiotic resistance genes (Figure 6A). However, the results from Resfams database searching identified more than 30 vancomycin resistance genes in the *L. plantarum* Y44 genome (Figure 6B). Although the EFSA guideline does not require vancomycin susceptibility of *L. plantarum*, MIC of vancomycin for *L. plantarum* Y44 was more than 128 $\mu\text{g/mL}$. This finding was consistent with previous research showing that *Lactobacillus* strains exhibited resistance to vancomycin (Ammor et al., 2007; Liu et al., 2009).

Lactobacillus, *Pediococcus*, and *Leuconostoc* species were reported to be resistant to vancomycin due to their possession of D-Ala-D-lactate in their peptidoglycan rather than the D-Ala-D-Ala dipeptide (Ammor et al., 2007). According to EFSA FEEDAP Panel et al. (2018), bacteria used as probiotics for humans or as additives for animal feed should not carry transferable antimicrobial resistance genes. The results from ResFinder showed that *L. plantarum* Y44 does not carry any acquired antibiotic resistance genes, indicating that the vancomycin resistance genes of *L. plantarum* Y44 were intrinsic rather than acquired by horizontal genomic transfer. Zhang et al. (2012) reported that the intrinsic type of antibiotic resistance of lactobacilli is not a particular safety concern.

Biogenic Amine Production

Biogenic amines compose a group of low-molecular-weight nitrogenous compounds that are produced through microbial amino acid decarboxylation (Capozzi et al., 2012). Dairy products, in particular cheese, can accumulate high levels of biogenic amines, mainly

Table 3. Antibiotic susceptibility of *Lactobacillus plantarum* Y44

Antibiotic	MIC ¹ (μg/mL)	Cut-off value ² (μg/mL)	Susceptibility ¹
Gentamicin	2	16	S
Kanamycin	64	64	S
Tetracycline	16	32	S
Clindamycin	2	2	S
Ampicillin	2	2	S
Erythromycin	0.25	1	S
Chloramphenicol	4	8	S
Vancomycin	NR	>128	NR

¹NR = not reported; S = susceptible.
²Cut-off value was based on European Food Safety Authority (EFSA) recommendations.

histamine and tyramine, which are known to be toxic (Ladero et al., 2012). Control of biogenic amines to be within the safety level is critical for the safety assessment of probiotics (Li et al., 2018). As shown in Figure 7, the decarboxylase medium plate inoculated with *L. plantarum* Y44 did not show a purple color, indicating that the strain did not produce biogenic amine. The genome sequencing results showed that *L. plantarum* Y44 has only one amino acid decarboxylase gene, glutamate decarboxylase (DQQ11_14585). Zhang et al. (2012) reported that the *L. plantarum* JDM1 genome contained glutamate decarboxylase, but the strain did not produce biogenic amine. Glutamate decarboxylase catalyzes α-decarboxylation of L-glutamate to produce 4-aminobutanoate (γ-aminobutyric acid), which was not involved in producing biogenic amines (Huang et al., 2018). The genotype of *L. plantarum* Y44 could

account for its phenotypic characteristic of not producing biogenic amines. Hill et al. (2017) recommended that preselection of starter cultures lacking the amino acid decarboxylase gene may be necessary to avoid unwanted build-up of biogenic amine compounds in fermented foods.

CONCLUSIONS

In this study, we investigated the physiological functions of *L. plantarum* Y44 by analyzing its phenotypic and genotypic characteristics. *Lactobacillus plantarum* Y44 could metabolize 24 different sugars. It also had BSH activity, the capacity to adhere to HT-29 cell monolayers, susceptibility to different antibiotics, no biogenic amine production, and antioxidant enzyme activity. The results of the *L. plantarum* Y44 genome

Figure 6. Predicted antibiotic resistance genes of *Lactobacillus plantarum* Y44: (A) CARD database prediction, and (B) Resfam database prediction.

Figure 7. Biogenic amine production of *Lactobacillus plantarum* Y44 with different precursor amino acids. *L. rhamnosus* = *Lactobacillus rhamnosus*.

analysis revealed gene clusters associated with carbohydrate transport and metabolism systems, proteolytic systems, amino acid metabolism, EPS biosynthesis, surface protein biosynthesis, stress response, and antibiotic resistance. The phenotypic characteristics of *L. plantarum* Y44 were associated with its genotypic characteristics. The physiological functions of *L. plantarum* Y44—such as tolerance to acid, bile salt, and oxygen; proteolytic capability; and ability to metabolize various carbohydrates and amino acids—form the foundation for its commercial application in dairy products.

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ORCID

- Yuan Gao <https://orcid.org/0000-0002-3931-4272>
 Yujun Liu <https://orcid.org/0000-0001-6114-7178>
 Heping Zhang <https://orcid.org/0000-0003-4329-8006>
 Guangqing Mu <https://orcid.org/0000-0001-9356-753X>
 Yanfeng Tuo <https://orcid.org/0000-0001-7255-3753>