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Identification of salt tolerance-related genes of *Lactobacillus plantarum* D31 and T9 strains by genomic analysis

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Abstract

Purpose: The aim of this study was to identify salt tolerance-related genes of *Lactobacillus plantarum* D31 and T9 strains, isolated from Chinese traditional fermented food, by genomic analysis.

Methods: Tolerance of *L. plantarum* D31 and T9 strains was evaluated at different stress conditions (temperatures, acid, osmolality, and artificial gastrointestinal fluids). Draft genomes of the two strains were determined using the Illumina sequencing technique. Comparative genomic analysis and gene transcriptional analysis were performed to identify and validate the salt tolerance-related genes.

Results: Both *L. plantarum* D31 and T9 strains were able to withstand high osmotic pressure caused by 5.0% NaCl, and *L. plantarum* D31 even to tolerate 8.0% NaCl. *L. plantarum* D31 genome contained 3,315,786 bp (44.5% GC content) with 3106 predicted protein-encoding genes, while *L. plantarum* T9 contained 3,388,070 bp (44.1% GC content) with 3223 genes. Comparative genomic analysis revealed a number of genes involved in the maintenance of intracellular ion balance, absorption or synthesis of compatible solutes, stress response, and modulation of membrane composition in *L. plantarum* D31 and or T9 genomes. Gene transcriptional analysis validated that most of these genes were coupled with the stress-resistance phenotypes of the two strains.

Conclusions: *L. plantarum* D31 and T9 strains tolerated 5.0% NaCl, and D31 even tolerated 8.0% NaCl. The draft genomes of these two strains were determined, and comparative genomic analysis revealed multiple molecular coping strategies for the salt stress tolerance in *L. plantarum* D31 and T9 strains.

Keywords: *Lactobacillus plantarum*, Salt stress tolerance, Genome sequence, Comparative genomics, Gene transcription, Traditional fermented food

Introduction

Lactic acid bacteria (LAB) are generally recognized as safe food-grade microorganisms. Numerous previous studies have revealed their beneficial effects on human health, such as maintaining the balance of gastrointestinal microbial community, acting against pathogenic microorganisms, and enhancing innate and adaptive immune responses (Chen et al. 2014; Liu et al. 2018; Nazir et al. 2018). *Lactobacillus plantarum* is one of the most widely applied LAB in the food industry. The bacterium is found to thrive in indigenous microbiota commonly found in fermented food (Zago et al. 2011) and can competitively inhibit pathogenic bacteria growth during fermentation (Molin 2001).

LAB chosen for commercial purposes must challenge adverse conditions encountered in industrial processes, such as heat, cold, acidity, and high concentrations of NaCl (Bucka-Kolendo and Sokolowska 2017). Many fermented food are made with the salt, by which osmotic stress is often a significant challenge for microorganisms surviving in fermentation processes (El-Gendy et al. 1983; Yamani et al. 1998; Prasad et al. 2003; Rao et al. 2004). Possible mechanisms of the regulation of intracellular osmotic pressure in LAB have been mentioned,

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such as (1) exclusion of Na^+ ion from cells, (2) accumulation of compatible solutes, and (3) changes of cell membrane composition. In the past decade, a number of LAB strains have been subjected for genome sequencing to further address their physiological functions and environmental adaptation mechanisms, along with the development of genome sequencing technologies. To date, more than 50 complete genome sequences of *L. plantarum* strains are available in the GenBank database (http://www.ncbi.nlm.nih.gov/genome/). Genomic diversity and versatility of *L. plantarum* have been reported (Siezen and van Hylckama Vlieg 2011; Jiang et al. 2018; Evanovich et al. 2019). Nevertheless, among these strains, only *L. plantarum* ST-III has been reported to be able to survive in De Man Rogosa and Sharp (MRS) medium with 7.5% NaCl (Chen et al. 2012). A *kdp* gene cluster encoding a high-affinity K^+-transport system was identified from a 53.56-kb plasmid pST-III in *L. plantarum* ST-III, which was found to contribute to its viability under hyperosmotic conditions (Chen et al. 2012). Recently, Wang et al. reported that *L. plantarum* ATCC 14917 was also able to survive in the MRS medium with 6.0% NaCl. The expression of eleven genes were upregulated in this bacterium to respond to the salt stress, including those involved in carbohydrate metabolism, transcription and translation, fatty acid biosynthesis, and primary metabolism (Wang et al. 2016).

L. plantarum with novel functional properties is of interest to both academic institution and food industry. In our prior studies, a number of LAB strains were isolated from Chinese traditional fermented food and identified and characterized by Xu et al. (Xu et al. 2016). Among these, *L. plantarum* D31 and *L. plantarum* T9 strains showed high levels of antioxidant and bile salt hydrolase activities in in vitro tests (Xu et al. 2016). In this study, tolerance of these two strains to various stress conditions was further evaluated, and the resulting data showed that both *L. plantarum* D31 and T9 strains were able to survive in the MRS medium with 5.0% NaCl, and *L. plantarum* D31 even tolerated to 8.0% NaCl. Thus, draft genome sequences of these two strains were determined using the Illumina sequencing technique in order to get genomic insights into possible molecular mechanisms of the salt tolerance of *L. plantarum*.

Materials and methods

L. plantarum strains and cultural conditions

L. plantarum D31 and T9 strains, isolated from Chinese traditional fermented food Dongbei kimichi and milk tofu, respectively, have been identified and characterized in our previous research (Xu et al. 2016). In this study, these two strains were individually inoculated from our laboratory stock at − 80 °C into the MRS medium (pH 6.8, Beijing Land Bridge Technology, Beijing, China) and incubated at 37 °C for 24 h under anaerobic conditions as described previously (Xu et al. 2016). Bacterial cells were harvested by centrifugation at 4000 r/min for 10 min and washed three times using the sterile 1 × phosphate-buffered saline (PBS, pH 7.0, Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., Shanghai, China). Then, cell pellets were resuspended with an appropriate amount of sterile deionized water and used as the inoculum (10^7–10^8 colony forming unit (CFU)/ml) in the further analyses. Cell density was determined using a multimode microplate reader (Synergy, BioTek Instruments, Winooski, VT, USA), and the OD_{600nm} value was used as a related parameter for the amount of bacteria biomass (Dahroud et al. 2016).

Stress conditions

L. plantarum D31 and T9 strains were individually incubated in the MRS medium (pH 6.8) at different temperatures (15 °C, 20 °C, 25 °C, 37 °C, and 45 °C) for 72 h, and their growth curves were measured according to the method described previously (Li et al. 2017). Acid tolerance of *L. plantarum* D31 and T9 strains was examined according to the method described previously (Lee et al. 2014) with slight modification. The cell suspension of *L. plantarum* D31 and T9 strains was individually inoculated into acidic MRS broth (pH 2.0 to 7.0) and incubated at 37 °C for 24 h. Tolerance of *L. plantarum* D31 and T9 strains to different concentrations of NaCl (0.0%, 5.0%, 8.0%, 10.0%, 12.0%, and 15.0%) was determined according to the method described previously (Xin et al. 2014). Bile salt tolerance of *L. plantarum* D31 and T9 strains was evaluated according to the method described previously (Shehata et al. 2016) with slight modification. The cell suspension of *L. plantarum* D31 and T9 strains was individually inoculated into the MRS broth containing different concentrations of bile salt (0.0%, 0.05%, 0.1%, 0.2%, and 0.3%) (Beijing Land Bridge Technology, Beijing, China) and incubated at 37 °C for 24 h. Growth curves of these two strains at different acid, osmotic pressure, and bile salt conditions were individually determined as described above. Tolerance of *L. plantarum* D31 and T9 strains to artificial gastric and intestinal fluids was determined according to the method described previously (Zhang et al. 2016) with slight modification. The cell suspension of *L. plantarum* D31 and T9 strains was individually inoculated (10%, v/v) into the artificial gastric and intestinal fluids and incubated at 37 °C for 180 min and 240 min, respectively. The viable cell count assay was performed as described previously (Zhang et al. 2016). Artificial gastric fluid contained 0.35% pepsin and 0.2% NaCl in 100 ml of distilled water. The solution pH was adjusted to 2.0 with 1 mol/l HCl (Zhang et al. 2016). Artificial intestinal fluid contained 0.1% trypsin, 1.1% $NaHCO_3$, and 0.2% NaCl in 100 ml of

distilled water. The solution pH was adjusted to 6.8 with 0.6 mol/l NaOH (Zhang et al. 2016). The artificial gastrointestinal fluids were sterilized by filtering through a 0.22-μm membrane (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., Shanghai, China).

Genomic DNA preparation

Genomic DNA was prepared using a MiniBEST DNA extraction kit (Japan TaKaRa BIO, Dalian Company, China) following the manufacturer's instructions. Extracted DNA samples were analyzed by electrophoresis with a 0.7% agarose gel and visualized and recorded using a UVPEC3 Imaging system (UVP LLC, UpLand, CA, USA) (Figure S1). The DNA concentration and purity (A_{260}/A_{280}) were measured using a multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT). Only pure genomic DNA samples (a 260/280 nm absorbance ratio of 1.8–2.0) were used for genome sequencing.

Genome sequencing and assembly

The genome sequencing of *L. plantarum* D31 and *L. plantarum* T9 strains was carried out at Meiji Biological Medicine technology Ltd. (Shanghai, China) and Beijing Novogene Bioinformatics Technology Co., Ltd. (Beijing, China) using a Genome Sequencer Illumina HiSeq Xten platform (Illumina, CA, USA), respectively. Sequence quality was analyzed using the FastQC software (Brown et al. 2017). Raw sequencing reads were trimmed and assembled using the SOAPdenovo v2.04 software (http://soap.genomics.org.cn/).

Genome annotation

Protein-encoding genes, tRNA genes, and rRNA genes were predicted using the GeneMarks (version 4.17) (Besemer et al. 2001) and Glimmer (version 3.02) (Delcher et al. 2007), tRNA_scan-SE (version 1.3.1) (Lowe and Eddy 1997), and RNAmmer (version 1.2) (Lagesen et al. 2007) software, respectively. Protein functions were predicted against the Clusters of Orthologous Groups (COG) database (Tatusov et al. 2001). Prophage-associated genes were predicted using a Prophage finder software (http://phast.wishartlab.com/). Clustered regularly interspaced short palindromic repeats (CRISPRs) were detected using the CRISPRFinder software (Grissa et al. 2007). Pfam domain, signal peptide and transmembrane domain, and transmembrane helices were predicted using the Web CD-Search Tool (Marchler-Bauer et al. 2015), SignalP 4.1 Serve (Petersen et al. 2011), and TMHMM (Krogh et al. 2001) software, respectively. Potential virulence factors were detected against the Virulence Factor Database (http://www.mgc.ac.cn/VFs/). Antibiotic resistance genes were searched in the Antibiotic Resistance Genes Database (Gupta et al. 2014).

Comparative genome analysis

Comparative genomic analysis was performed between *L. plantarum* D31 and T9 strains, and 50 *L. plantarum* strains whose complete genome sequences (Table S1) were available by 31 March 2018. The complete genome sequences were retrieved from the NCBI genome database (http://www.ncbi.nlm.nih.gov/genome). The Blastcluster software (http://www.ncbi.nlm.nih.gov/) was used for pan-genome analysis. Orthologous genes were analyzed using the CD-HIT software (Fu et al. 2012). Orthologous proteins were assigned only for proteins sharing both 60% amino acid identity and 80% sequence coverage, and strain-specific genes present in one genome had no significant BLAST hit against reference groups at $E \leq 1e^{-5}$. Homologous sequences of each gene were aligned using the MUSCLE software (Edgar 2004). A phylogenetic tree was constructed and viewed using the PHYML (Guindon and Gascuel 2003) and EvolView (Zhang et al. 2012) software, respectively. Bootstrap values above 50% were obtained from 1000 bootstrap replications.

Quantitative real-time reverse transcription PCR (qRT-PCR)

Selected salt resistance-associated genes were validated by qRT-PCR assay as described previously (Sun et al. 2014; Zhu et al. 2017). *L. plantarum* D31 and T9 strains were incubated in the MRS medium supplemented with 8% and 5% NaCl, respectively, and cell culture grown to logarithmic growth phase was harvested by centrifugation as described above. Total RNA was prepared using the RNeasy Protect Bacterial Mini Kit (QIAGEN Biotech Co., Ltd., Hilden, Germany) according to the manufacturer's instructions. The DNA was removed from the samples using RNase-Free DNase Set (QIAGEN, Hilden, Germany). RNA samples were analyzed by 1% agarose gel electrophoresis at 100 V for 30 min (Figure S2), and its quality and quantity was assessed using a multi-mode microplate reader (BioTek Instruments, Inc., Winooski, VT). The reverse transcription reaction was performed using the PrimeScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (Japan TaKaRa BIO, Dalian Company, Dalian, China) according to the manufacturer's protocol. RT-PCR was performed using the TB Green® Premix Ex Taqt™ II (Tli RNaseH Plus) (Japan TaKaRa BIO, Dalian Company, Dalian, China) according to the manufacturer's instructions. A 20-μl reaction volume contained 10 μl TB Premix Ex TaqTM II, 0.4 μl of each of the oligonucleotide primers (10 μmol), 0.4 μl of ROX Reference DyeII, 2 μl of cDNA template, and appropriate volume of sterile DNase/RNase-Free deionized water. All RT-PCR reactions were performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: initial denaturation at 95 °C for 10 min, followed by 40 cycles

of denaturation at 95 °C for 15 s, and primer annealing at 60 °C for 60 s. The 16S rRNA gene was used as the reference gene, as previously described (Zhu et al. 2017). The expression of the 16S rRNA gene in *L. plantarum* D31 and T9 strains grown to the logarithmic growth phase in MRS medium supplemented with no NaCl was used as a reference/baseline, respectively. The data were analyzed using the Applied Biosystems 7500 software, and the relative expression ratio was calculated for each target gene by using the delta-delta threshold cycle (Ct) method (Livak and Schmittgen 2001). Oligonucleotide primers were designed using the Primer 5.0 software (http://www.premierbiosoft.com/) and synthesized by Shanghai Sangon Biological Engineering Technology Services Co. Ltd. (Shanghai, China) (Table 1). All determinants were performed in triplicate.

Genome sequence accession numbers

The draft genomes of *L. plantarum* D31 and *L. plantarum* T9 strains were deposited in GenBank under the accession numbers RCFP00000000 and RBAI00000000, respectively.

Results and discussion

To date, more than 50 complete genome sequences of *L. plantarum* strains are available in public databases; nevertheless, little genome information is for the salt-tolerant LAB. Moreover, only few *L. plantarum* strains have been reported to tolerate osmotic stress, e.g., *L. plantarum* ST-III and *L. plantarum* ATCC 14917 strains (El-Gendy et al. 1983; Rao et al. 2004; Chen et al. 2012; Vasyliuk et al. 2014; Xin et al. 2014). In this study, for the first time, we evaluated tolerance of *L. plantarum* D31 and *L. plantarum* T9 strains, isolated from Chinese traditional fermented food, to different stress conditions (temperatures, acid, osmolality, and artificial gastrointestinal fluids).

Survival of *L. plantarum* D31 and T9 strains at different temperatures

Growth curves of *L. plantarum* D31 and T9 strains incubated at different temperatures (15 to 45 °C) were determined, and the resulting data are illustrated in Fig. 1a–c. No distinct difference in growth was observed between these two strains at an optional growth temperature of 37 °C (Fig. 1c). Moreover, at the lower (15 °C) or higher (45 °C) temperatures, the growth of these two strains was obviously inhibited, and their OD_{600nm} values showed no significant change for 72 h (figure not shown). Nevertheless, when incubated at 20 °C, *L. plantarum* T9 still grew well but with a long lag phase (18 h) and reached stationary growth phase (SGP) at 54 h, whereas the growth of *L. plantarum* D31 was retarded with a longer lag phase (54 h) and entered into the SGP at 72 h (Fig. 1a). Similar growth phenotypes of these two strains were observed at 25 °C as did at 20 °C (Fig. 1b), suggesting the medium-temperature growth feature of *L. plantarum* T9 and D31 strains.

Survival of *L. plantarum* D31 and T9 strains at acidic pH conditions

Tolerance of *L. plantarum* under acid stress has been reported (Huang et al. 2016). In this study, as shown in Fig. 2, *L. plantarum* T9 and D31 strains were able to grow at pH 5.0 and optimally at pH 6.0 to 7.0, consistent with previous research (Nyanga-Koumou et al. 2012). Additionally, *L. plantarum* D31 did not grow at pH 4.0, whereas *L. plantarum* T9 was observed to grow slowly at this pH condition. Moreover, no cell growth of these two strains was observed under more acidic conditions with pH values lower than 3.0 (Fig. 2).

Tolerance of *L. plantarum* D31 and T9 strains to different concentrations of NaCl

Tolerance of *L. plantarum* D31 and T9 strains to different concentrations of NaCl was determined, and the resulting data are illustrated in Fig. 3. The two strains were found to grow well in the MRS medium supplemented with 0.0–5.0% NaCl at 37 °C. Also, *L. plantarum* D31 was able to grow at 8.0% NaCl, whereas the growth of *L. plantarum* T9 was obviously inhibited at this NaCl concentration. No cell growth of these two strains was found when the NaCl concentration was more than 8.0% (Fig. 3).

Survival of *L. plantarum* D31 and T9 strains in different concentrations of bile salt

Previous research has reported bile-tolerant *L. plantarum* and key proteins by comparative proteomic analysis (Hamon et al. 2011). In this study, as shown in Fig. 4, bile salt tolerance of *L. plantarum* D31 and T9 strains was examined. The growth of these two strains was obviously inhibited at 0.05% bile salt. Moreover, neither *L. plantarum* D31 nor T9 strains could withstand more than 0.1% bile salt (Fig. 4).

Survival of *L. plantarum* D31 and T9 strains in artificial human gastric and intestinal fluids

Tolerance of *L. plantarum* D31 and T9 strains to artificial gastric and intestinal fluids was also determined. After treated in the artificial gastric fluid for 180 min, the growth of *L. plantarum* D31 was completely inhibited, while an extremely low survival rate (0.03%) was observed for *L. plantarum* T9. Likewise, the growth of *L. plantarum* D31 and T9 strains was also significantly reduced in artificial intestinal fluid for 240 min, and the survival rates were 11.7% and 0.87% for *L. plantarum* D31 and T9 strains, respectively (figures not shown).

Table 1 The oligonucleotide primers used in the qRT-PCR assay in this study

Locus/gene	Description of encoded protein	Sequence (5′– > 3′)	Predicted product length (bp)	Source
L. plantarum D31				
D7Y65_00120	Potassium transporter Kup	F: ATCGTTGGCTCTTTAATCCC	166	This study
		R: AACAAGCACGAAGGCGGTAT		
D7Y65_03505	Cell membrane protein	F: TGTGACTGGGTTTGCTGAAT	124	This study
		R: CTCCAACACCGCATCTTCTA		
D7Y65_06915	Co-chaperone GroES	F: ATGACGGTCGTGTTTTAGAT	117	This study
		R: CCTTGATACTTGACTTCGGT		
D7Y65_06920	Chaperonin GroEL	F: GCTTCTGTTTCAGCCCTTCT	106	This study
		R: TACATCATACCGCCCATACC		
D7Y65_06955	Sigma-54 modulation protein	F: CAAGCAATTCGGGACTACGT	113	This study
		R: TTTAGCCGTCTTGTCTGGGT		
D7Y65_09830	Molecular chaperone DnaJ	F: TGGCGTATTAGTCTGTACCTG	164	This study
		R: TGAAGTTTGAAGATGCGATC		
D7Y65_09835	Molecular chaperone DnaK	F: ACCAAGTGAAAGTGGCGTAA	139	This study
		R: AAAGTGGACTGGCAAAGAAT		
D7Y65_13150	Potassium transporter Kup	F: GGACACGAAAGCCCAGGTAT	145	This study
		R: TTAGACAAGTGGCCGAAACG		
D7Y65_13295	LysR family	F: TGAAGGCCGTATTATGGATG	149	This study
		R: GATTGCCGAAGAATTTGACC		
*D7Y65_02550**	Metal-independent α-mannosidase	F: AGATACGGGAATGATTTGGT	182	This study
		R: TACGCACAGTCGTCTGGAGT		
*D7Y65_07055**	Glycosyl hydrolase family 8	F: CGGTGAGTGTTGGTGGTGAA	137	This study
		R: CGGCGAACTGTCTTGCTGTA		
*D7Y65_13825**	Carboxypeptidase	F: ATAATAGTGCGGATTGTGCT	181	This study
		R: TACTACCGTGACGATGGGAC		
*D7Y65_15155**	Potassium transporter Kup	F: AGCAGATGGCACCCTAACAC	169	This study
		R: AACGACGGCAGTACCAAACC		
*D7Y65_15160**	Kdp E	F: CTGCACTTTGTCAGCGTCCTC	104	This study
		R: CCGATATGGATGGGATGGAC		
*D7Y65_15165**	KdpD	F: TTTGGTTACGCTGCTTTCTT	106	This study
		R: TAACATTAGCCTTGCCCATC		
*D7Y65_15180**	Kdp A	F: AACCACCAGTTGTTTGAGGA	142	This study
		R: GGTATCCAACGTACAGAGGC		
L. plantarum T9				
D7Y66_02210	Cell membrane protein	F: TACCGGCTGCTTATGATACTTC	173	This study
		R: CTCCAACACCGCATCTTCTA		
D7Y66_03635	Co-chaperone GroES	F: CAATGACGGTCGTGTTTTAG	108	This study
		R: CCTTGATACTTGACTTCGGT		
D7Y66_03640	Chaperonin GroEL	F: GCTTCTGTTTCAGCCCTTCT	113	This study
		R: TACATCATACCGCCCATACC		
D7Y66_03675	Sigma-54 modulation protein	F: CAAGCAATTCGGGACTACGT	115	This study
		R: TAGCCGTCTTGTCTGGGTGA		
D7Y66_07525	Potassium transporter Kup	F: CTCAGCTATTTCGGTCAGGC	103	This study
		R: CCATCGTCAGGTTAGCAGGT		

Table 1 The oligonucleotide primers used in the qRT-PCR assay in this study *(Continued)*

Locus/gene	Description of encoded protein	Sequence (5′– > 3′)	Predicted product length (bp)	Source
D7Y66_08075	Molecular chaperone DnaJ	F: CCAGCAGGAACCGTCACTTT	140	This study
		R: CCACTCGGTCGAATGATGAG		
D7Y66_08080	Molecular chaperone DnaK	F: ACCAAGTGAAAGTGGCGTAA	139	This study
		R: AAAGTGGACTGGCAAAGAAT		
D7Y66_11330	LysR family	F: GGTTTCGAGACCGGCTAATA	179	This study
		R: GCAACTTGCTGAACACGCTA		
D7Y66_13435	Potassium transporter Kup	F: GGCAGTACCAAACCCTTGAA	168	This study
		R: TTGTTAGCAGATGGCACCCT		
lp_3505	Acetyl esterase	F: TTCGTGTTGGTAACTCGTGGGT	117	This study
		R: TGTTGCATGGCTTAGGTGGG		
16S RNA		F: AAGGGTTTCGGCTCGTAAAA	247	Sun et al. 2014
		R: TGCACTCAAGTTTCCCAGTT		

F forward primer, *R* reverse primer; *The genes detected in *L. plantarum* D31 strain

Genome features of *L. plantarum* D31 and T9 strains

Draft genomes of *L. plantarum* D31 and T9 strains were determined using the Illumina sequencing technique in order to get insights into possible molecular mechanisms of their salt stress tolerance. This analysis generated 8,987,722 and 6,246,667 reads for *L. plantarum* D31 and T9 with sequencing depth of 406-fold and 277-fold, respectively. The *L. plantarum* D31 draft genome contains 3,315,786 bp with a GC content of 44.5%. The final assembly comprised 72 scaffolds. Total 3251 genes were predicted, including 3106 predicted protein-coding genes and 48 RNA genes (Table 2). Among these genes, approximately 75.4% had a predicted function, and 58.3% were assigned to COG (Table S2). One 39.9-kb intact prophage element (scaffold 6: 70,583–110,507 bp) and one CRISPR repeat array (23 bp, scaffold 50: 77–343 bp) were identified in *L. plantarum* D31 genome.

The *L. plantarum* T9 draft genome contained 3,388,070 bp with a GC content of 44.1%. The final assembly yielded 158 scaffolds. Total 3515 genes were predicted, including 3223 predicted protein-coding genes and 77 RNA genes (Table 2). Among the predicted genes, about 73.7% had a predicted function, and 55.1% were assigned to COG (Table S2). Two intact prophage elements (76.5 kb, scaffold 1: 73,403–149,937 bp; 40.8 kb, scaffold 6: 16,933–57,799 bp) and one CRISPR repeat array (37 bp, scaffold 52: 11,856–12,956 bp) were identified in *L. plantarum* T9 genome, respectively.

Additionally, no virulence gene was identified in *L. plantarum* D31 and T9 draft genomes. A potential antibiotic gene *baca* encoding a bacitracin resistance protein (D7Y65_11270, D7Y66_07705) was identified in the two genomes.

The features of these two draft genomes are summarized in Table 2. The draft genomes of *L. plantarum* D31 and T9 were submitted to GenBank under the accession numbers RCFP00000000 and RBAI00000000, respectively.

Phylogenetic relatedness of *L. plantarum* strains

As shown in Fig. 5, a phylogenetic tree was construed, based on 151,630 homologous amino acid sequences

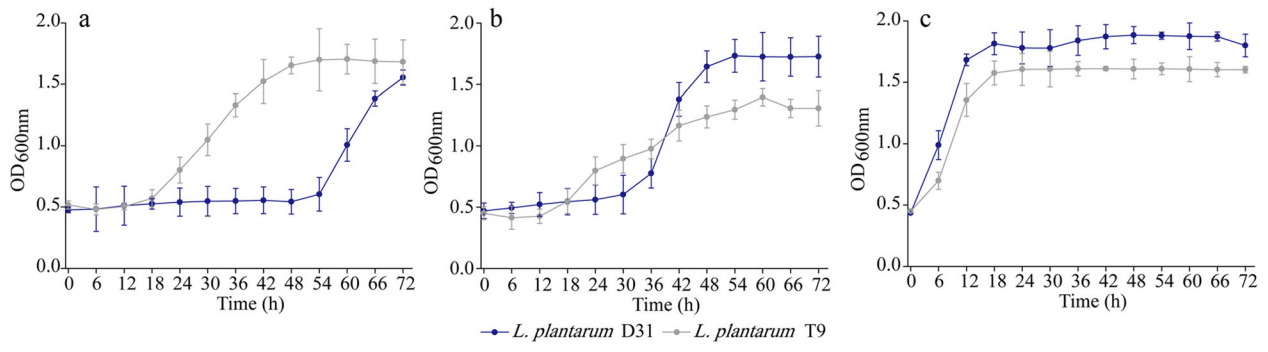

Fig. 1 Growth curves of *L. plantarum* D31 and T9 strains incubated in the MRS medium (pH 6.8) at different temperatures. **a** 20 °C, **b** 25 °C, and **c** 37 °C

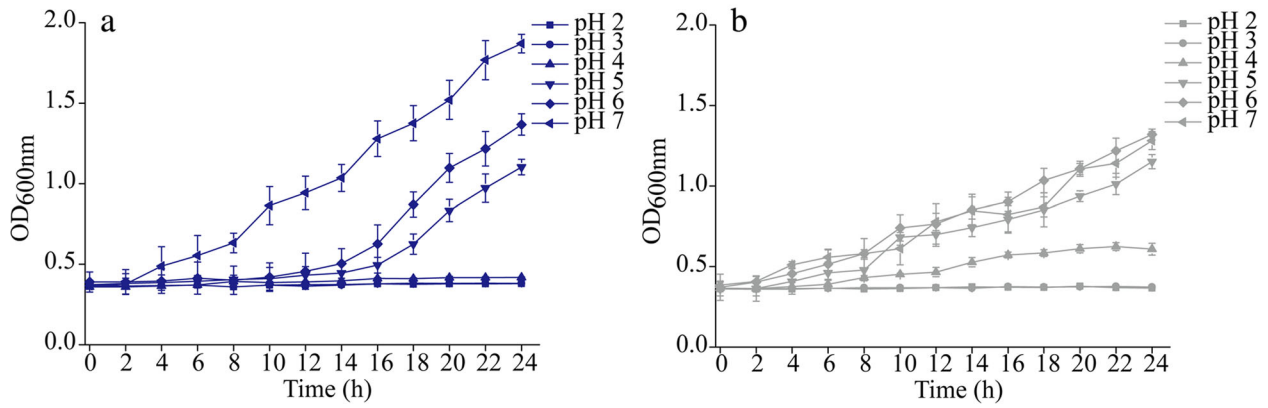

Fig. 2 Effects of different initial pH on the growth of *L. plantarum* D31 (**a**) and T9 (**b**) strains

identified from the 52 *L. plantarum* genomes analyzed in this study, among which complete genome sequences of 50 *L. plantarum* strains were available and retrieved from the GenBank database. This analysis revealed three distinct clusters, designated cluster α, β, and γ. *L. plantarum* D31 and T9 genomes were classified as two singletons (cluster α and cluster β). They were phylogenetically distant from the other *L. plantarum* genomes that were grouped into cluster γ. The cluster γ was further classified into two subclusters I and II, including 13 and 36 genomes, respectively, which were recovered from diverse sources, such as the human saliva and gut, fermented fish, pickle, stinky tofu, and cow milk. Additionally, *L. plantarum* D31 and T9 genomes were distant from the salt-tolerant *L. plantarum* ST-III (ASM14881V1).

Strain-specific genes in *L. plantarum* D31 and T9 genomes

Based on the 52 *L. plantarum* genome sequences analyzed in this study, comparative genomic analysis revealed 173 strain-specific genes in *L. plantarum* D31 genome, of which 167 genes encoded hypothetical proteins, suggesting possible strain-specific mechanisms of stress tolerance and/or niche adaptation. The remaining strain-specific genes were involved in cell wall biosynthesis, carbohydrate metabolism, and stress response, e.g., the L-fructose isomerase (D7Y65_15415), pilus assembly protein (D7Y65_15865), bleomycin binding protein Ble-MBL (D7Y65_15890), molecular chaperone DnaJ (D7Y65_15980), single-stranded DNA-binding protein (D7Y65_16040), and conjugal transfer protein TraG (D7Y65_16080). Likewise, *L. plantarum* T9 had 112 strain-specific genes; however, most of which (111 genes) encoded hypothetical proteins, and one encoded a helix-turn-helix domain-containing protein (D7Y66_00865).

Genomic insights into possible mechanisms of the salt tolerance of *L. plantarum* D31 and T9 strains

Recovery of intracellular ion balance

The sodium/proton (Na^+/H^+) reverse transporter on cytoplasma membrane is the main way of microbial efflux of Na^+, which regulates intracellular pH homeostasis (Padan et al. 2005). It has been reported that *L. plantarum* 5-2 genome contained eight genes encoding

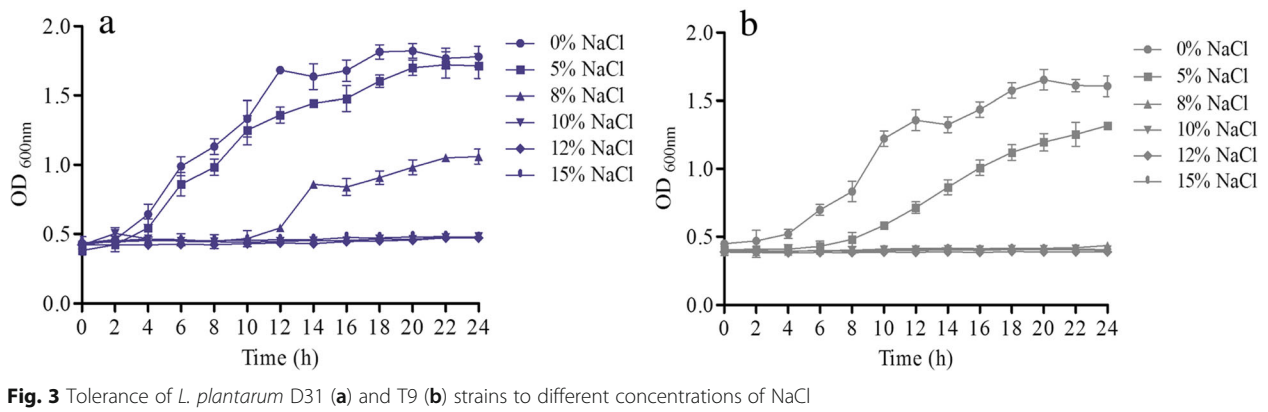

Fig. 3 Tolerance of *L. plantarum* D31 (**a**) and T9 (**b**) strains to different concentrations of NaCl

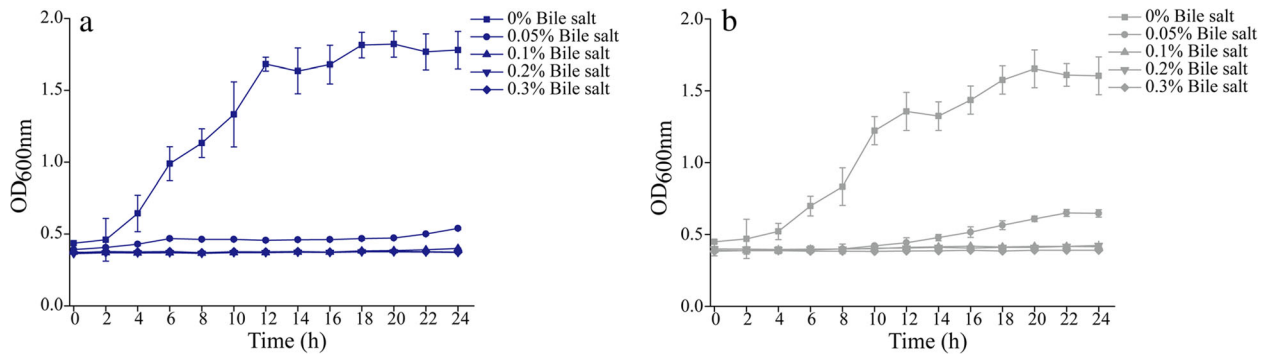

Fig. 4 Survival of *L. plantarum* D31 (**a**) and T9 (**b**) strains in different concentrations of bile salt

the Na^+/H^+ antiporters (Liu et al. 2015). In this study, comparative genomic analysis revealed at least ten, nine, and six genes encoding Na^+/H^+ antiporters in *L. plantarum* D31, T9, and ST-III genomes, respectively. Potassium (K^+) is the most abundant ion in bacterial cytoplasm and plays a pivotal role in ion homeostasis (Epstein 2003). Previous research has indicated that the *kdp* system in *L. plantarum* ST-III enabled the bacterium growing in the presence of curing salts (7.5% NaCl) (Chen et al. 2012). In the *kdp* system, the sensor kinase KdpD and the response regulator KdpE controlled the induction of the *kdpABC* operon in response to an osmotic upshift (Peddie et al. 1994; Grissa et al. 2007; Petersen et al. 2011). In this study, a typical *kdpABCDE* gene locus was also identified in *L. plantarum* D31 genome (D7Y65_15160 to D7Y65_15180), which had high sequence similarity (95%) with *kdp* genes (YP_003927890.1 to YP_003927894.1) in *L. plantarum* ST-III genome. The *kdp* cluster was also identified in another *L. plantarum* GB-LP3 genome, but absent from *L. plantarum* T9 draft genome. It has also been reported that K^+ is accumulated far above the normal level in the primary response in *Escherichia coli* to the osmotic upshift (Heermann et al. 2009). In *E. coli*, *kup* is the major K^+ uptake system under hyperosmotic stress and low pH conditions (Zakharyan and Trchounian 2001). In this study, upstream of the *kdp* gene cluster, a K^+-transport system gene *kup* was identified in *L. plantarum* D31 genome (D7Y65_15155), showing 94% sequence similarity with *kup* genes (WP_013356293.1) in *L. plantarum* ST-III, which may act as the major K^+ uptake system in the MRS medium with 7.5% NaCl (Chen et al. 2012). Moreover, another two *kup* genes (D7Y65_13150, D7Y65_00120) were also identified from *L. plantarum* D31 genome, while only two were identified from *L. plantarum* T9 (D7Y66_07525, D7Y66_13435).

Table 2 *L. plantarum* D31 and T9 genome statistics

Feature	*L. plantarum* D31		*L. plantarum* T9	
	Value	Percentage of total	Value	Percentage of total
Genome size (bp)	3,315,786	100.00	3,388,070	100.00
DNA coding (bp)	2,777,217	83.76	2,788,032	82.29
DNA G + C (bp)	1,474,530	44.47	1,494,478	44.11
DNA scaffold	72		158	
Total gene	3251	100.00	3515	100.00
Protein-coding gene	3106	95.54	3223	91.69
RNA gene	48	1.48	77	2.19
Pseudo gene	97	2.98	215	6.12
Genes with function prediction	2450	75.36	2590	73.68
Genes assigned to COG	1896	58.32	1935	55.05
Genes with Pfam domain	2530	77.82	2578	73.34
Genes with signal peptide	138	4.24	143	4.07
Genes with transmembrane helices	845	25.99	876	24.92
CRISPR repeat	1		1	
Intact prophage	1		2	

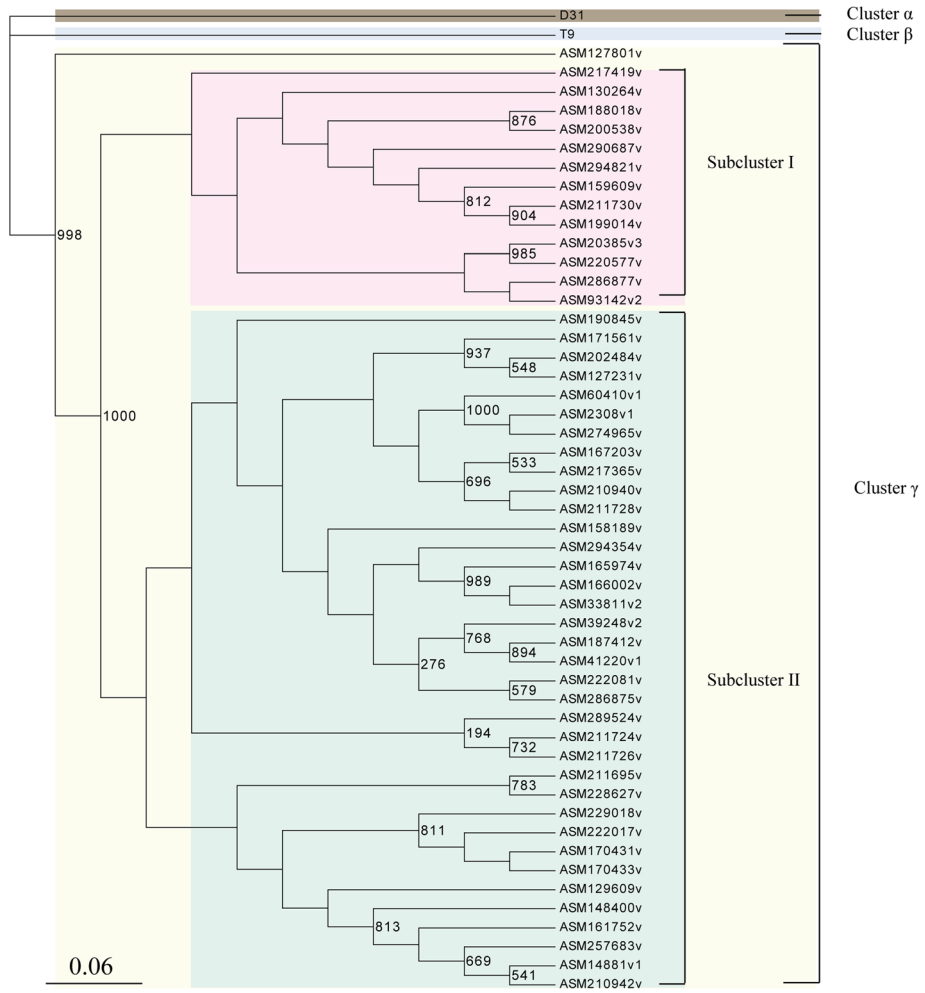

Fig. 5 Phylogenetic analysis of the 52 *L. plantarum* genomes analyzed in this study. The draft genome sequences of *L. plantarum* D31 and T9 were obtained in this study, while the complete genome sequences of the other 50 *L. plantarum* strains were available in the NCBI genome database

Absorption or synthesis of compatible solutes

Accumulation of certain compatible solutes (e.g., glycine and betaine) is a common metabolic adaptation found in diverse species (Oshone et al. 2017). The osmotic function of a compatible solute depends on the degree of methylation and length of the hydrocarbon chain (Peddie et al. 1994). It has been reported that the electrolyte-mediated osmolality up-shifts led to the accumulation of compatible solutes (Glaasker et al. 1998). In this study, genes involved in absorption or synthesis of compatible solutes were identified in *L. plantarum* D31 and T9 genomes. For instance, the genes encoding glycine/betaine/carnitine ABC transporters (*opuABCD*, *choSQ*) were identified in *L. plantarum* D31 (D7Y65_03265 to D7Y65_03280) and T9 (D7Y66_01970 to D7Y66_01985) genomes, respectively, which had high sequence similarity (99%) with the corresponding genes in *L. plantarum* ST-III (Kleerebezem et al. 2003). Moreover, the gene involved in nitrate/sulfonate/bicarbonate ABC transporter was identified in *L. plantarum* D31 (D7Y65_10050) and T9 genomes (D7Y66_08295), respectively, which was upregulated in response to salt stress in *L. plantarum* ST-III (Kleerebezem et al. 2003). These genes were involved in multi-component binding-protein-dependent transport systems for glycine, betaine, and camitine, and accumulated to high levels in the cell in response to increased external osmolality (Huang et al. 2010). Additionally, proline is essential for primary metabolism in salt stress and plays a molecular chaperone role in maintaining the pH of the cytosolic redox status of the cell (Kido et al. 2013). Previous research has indicated that *proABC* genes are related to the accumulation of proline and enable bacteria to withstand high osmotic pressure (Mahan and Csonka 1983). In this study, a *proABC* gene cluster was identified in *L. plantarum* D31 (D7Y65_02230, D7Y65_02235, D7Y65_14760) and T9

(D7Y66_05380, D7Y66_05385, D7Y66_11000) genomes, respectively, which may be involved in their tolerance to the salt stress.

Modulation of cell membrane

The composition of the cell envelope plays an important role in bacterial osmo-adaptation (Sun et al. 2014). Salt stress triggers alterations in structure and composition of the cell peptidoglycan layer (Piuri et al. 2010). In this study, comparative genomic analysis also revealed the genes involved in the modulation of cell membrane in *L. plantarum* D31 and T9 genomes. For instance, the genes encoding a lysyl-phosphatidylglycero (D7Y65_14200, D7Y66_03470) and a phosphatidylglycero (D7Y65_14200/D7Y65_07030, D7Y66_03470/D7Y66_15490) were identified in *L. plantarum* D31 and T9 genomes, respectively. Moreover, the gene encoding a membrane protein (D7Y65_03505, D7Y66_02210) was also identified in *L. plantarum* D31 and T9 genomes, which had 50% sequence similarity with the gene (CCI6_RS12035) in *Frankia* sp. Ccl6, which was involved in cell wall/membrane/envelop biosynthesis and upregulated under the salt stress (Oshone et al. 2017). *L. plantarum* D31 and T9 also contain the genes encoding a 1-acylglycerol-3-phosphate O-acyltransferase (D7Y65_09990, D7Y66_08235) and a phosphatidylglycerophosphatase A (D7Y65_11075, D7Y66_07900), which are involved in cell wall modification to response to the salt stress in *Frankia* strains (Oshone et al. 2017).

Stress response

Previous studies have indicated that induction of one-component regulatory systems (e.g., GroES-GroEL and DnaK-DnaJ) is related with acid, ethanol, cold, osmotic, starvation, and temperature stresses (Sugimoto et al. 2008). DnaK was first found in heat stress in *E. coli* (Arsene et al. 2000), but it can also be overexpressed under salt stress (Bucka-Kolendo and Sokolowska 2017). In this study, the genes encoding one-component regulatory systems DnaK-DnaJ and GroES-GroEL were identified in *L. plantarum* D31 (D7Y65_09835 to D7Y65_09830, D7Y65_06915 to D31_D7Y65_06920) and T9 genomes (D7Y66_08080 to D7Y66_08075, D7Y66_03635 to D7Y66_15720), respectively. A gene encoding GroES-like protein (D7Y65_06915) was also identified in *L. plantarum* D31 and ATCC14917 genomes. Qin et al. reported that a marine bacterium *Zunongwangia profunda* (MCCC 1A01486) showing extreme salt tolerance has a cold-active and salt-tolerant α-amylase (AmyZ) belonging to glycoside hydrolase family 13 (Qin et al. 2014). Kang et al. also reported that the gene lj0569 encoding a conserved domain of glycoside hydrolase family 31 was present in *Lactobacillus johnsonii* NCC533, which was found to survive in a high concentration of NaCl (29 g/l) (Kang et al. 2009). The genes encoding proteins that

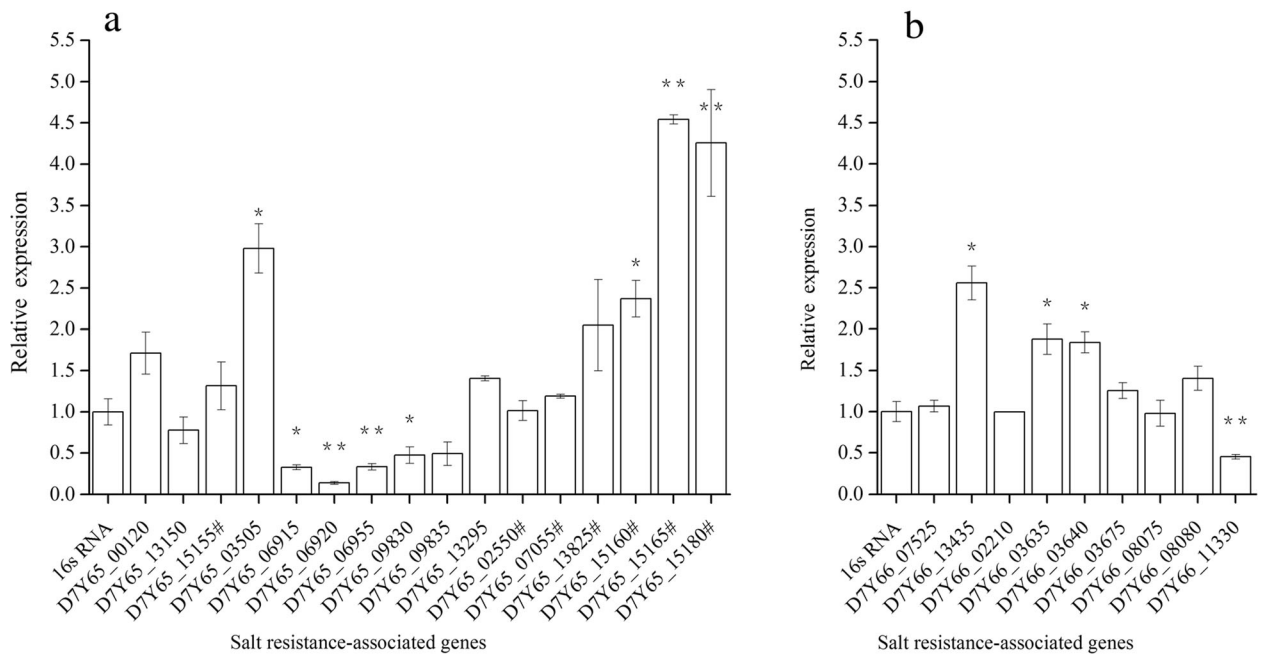

Fig. 6 Expression of salt resistance-associated genes in *L. plantarum* D31 (**a**) and T9 (**b**) strains in the salt stress conditions by qRT-PCR assay. *L. plantarum* D31 and T9 strains were incubated to the logarithmic growth phase in 8% and 5% NaCl, respectively. #Salt resistance-associated genes identified in *L. plantarum* D31 genome. *$p < 0.05$. **$p < 0.01$

have conserved domains of glycoside hydrolase families 125 and 8 were identified in *L. plantarum* D31 genome (D7Y65_02550, D7Y65_07055), which had 99% sequence similarity with corresponding genes (WP_013356072.1, WP_033099061.1) in salt-tolerant *L. plantarum* ST-III.

Regulators

Previous studies have revealed important roles of regulators in stress tolerance of *L. plantarum* (Wang et al. 2016; Jia et al. 2018). *L. plantarum* D31 draft genome contained approximately 229 genes encoding transcriptional or response regulators, which represented approximately 7.4% of its protein-encoding genes, while *L. plantarum* T9 contained approximately 208 such genes, which represented approximately 6.5% of its protein-encoding genes. These genes may modulate global regulatory networks that are essential for bacterial adaptation to changing environment. For instance, several genes encoding transcriptional factors of GntR, TetR, Crp/Fnr, and LysR families were identified in *L. plantarum* D31 and T9 genomes (Table S3), which have been implicated in bacteria stress responses including heat and osmotic shock (Ramos et al. 2005). The gene (D7Y65_13295, D7Y66_11330) encoding transcriptional regulator of the LysR family was identified in *L. plantarum* D31 and T9 genomes, which shared 61.1% similarity at amino acid sequence level with the gene (CCI6_RS20460) in a salt stress-tolerant *Frankia* sp. Ccl6 strain. Genes encoding a RNA polymerase sigma factor RpoD (D7Y65_04790, D7Y66_09275), an S-adenosylmethionine synthetase (D7Y65_12145, D7Y66_14505), a DNA-directed RNA polymerase subunit beta (D7Y65_08230, D7Y66_06445), and an amino acid permease (D7Y65_06925, D7Y66_03645) were also identified in *L. plantarum* D31 and T9 genomes, respectively. The expression of these genes was upregulated in *Frankia* sp. Ccl6 grown in 1000 mmol/l NaCl (Oshone et al. 2017). In addition, many differentially expressed proteins responding to 6.0% NaCl stress were identified in *L. plantarum* ATCC14917 (Wang et al. 2016), among which the genes encoding a triosephosphate isomerase (D7Y65_07215, D7Y66_03905), a glyceraldehyde-3-phosphate dehydrogenase (D7Y65_07205, D7Y66_03895), a fructose-bisphosphate aldolase (D7Y65_00895, D7Y66_11760), a trigger factor (D7Y65_10490, D7Y66_10765), a carbamoyl phosphate synthase large subunit (D7Y65_06465, D7Y66_02930), an orotate phosphoribosyltransferase (D7Y65_

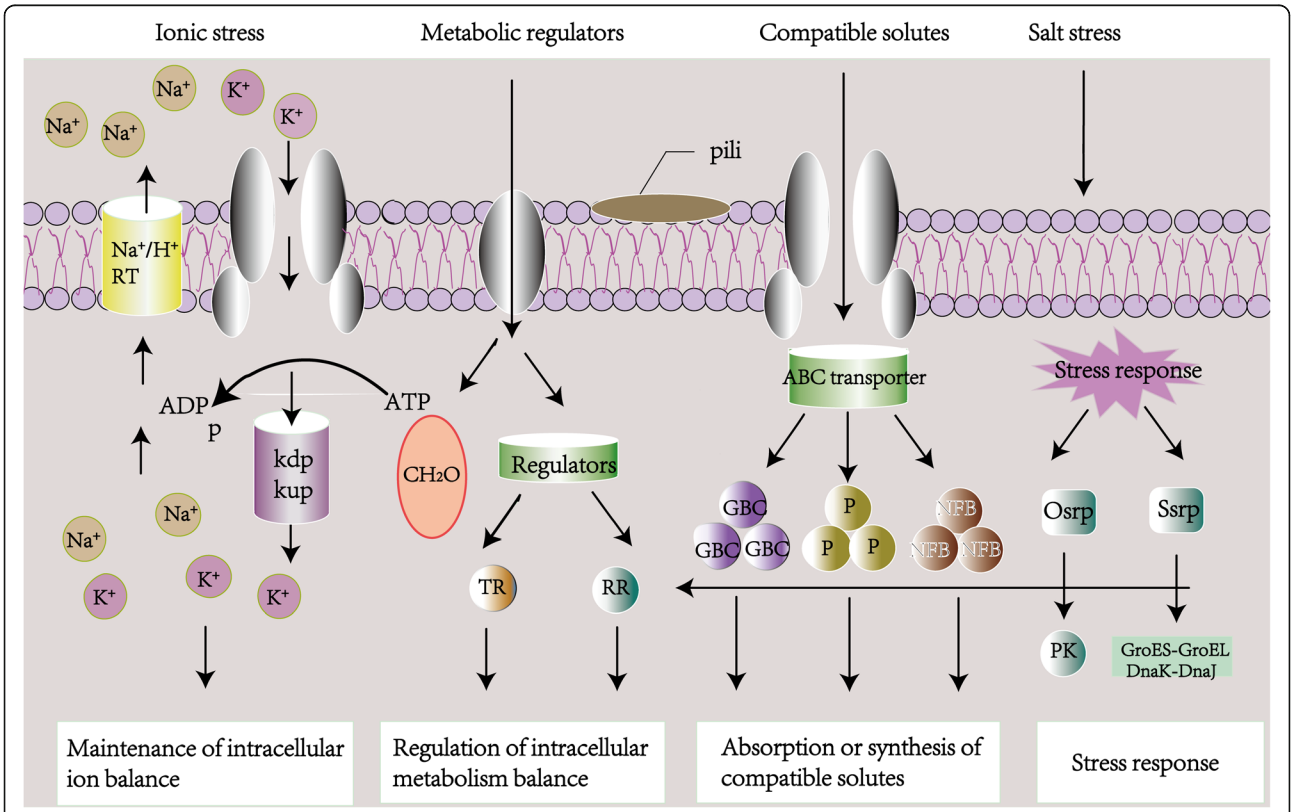

Fig. 7 Possible salt tolerance mechanisms of *L. plantarum* D31 and T9 strains. CH_2O, carbohydrate metabolism; GBC, glycine/betaine/carnitine ABC transporters; kdp, kup, K^+ transport systems; Na^+/H^+ RT, Na^+/H^+ reverse transport; NFB, nitrate/sulfonate/bicarbonate ABC transporters; Osrp, oxidative stress-related proteins; P, proline; pili, pilus assembly proteins; PK, protein kinases; RR, response regulators; Ssrp, salt stress-related proteins; TR, transcription regulators

06450, D7Y66_02945), an elongation factor Tu (D7Y65_10495, D7Y66_10760), a glutamyl-tRNA synthase (D7Y65_14120, D7Y66_03390), a S-ribosylhomocysteine lyase (D7Y65_07140, D7Y66_03830), a malonyl CoA-acyl carrier protein transacylase (D7Y65_03550, D7Y66_02255), and a sigma-54 modulation protein (D7Y65_06955, D7Y66_03675) were also identified in *L. plantarum* D31 and T9 genomes, respectively. In addition, the gene encoding a carboxypeptidase was identified in *L. plantarum* D31 (D7Y65_13825) and ST-III, which shared 25% amino acid sequence similarity with the protein (WP_010874021.1) directly or indirectly involved in salt sensing of *Synechocystis* sp. PCC 6803 (Huang et al. 2010).

Transcriptional profiles of salt resistance-associated genes in *L. plantarum* D31 and T9 strains

To couple the salt tolerance phenotypes of *L. plantarum* D31 and T9 strains with their salt resistance-associated genes identified by the comparative genomic analysis, we determined transcriptional profiles of 16 representative genes by the qRT-PCR assay. This analysis revealed many differentially expressed genes involved in the salt stress in the two strains (Figure S3, Fig. 6). For instance, when *L. plantarum* D31 was grown in 8% NaCl, the expression of the *kdp* gene cluster and its regulators (*D7Y65_15160 D7Y65_15165 D7Y65_15180*) was remarkably upregulated (2.4–4.5 fold, $p < 0.05$), suggesting enhanced K^+ uptake of *L. plantarum* D31 cells in the stress condition. Likewise, when *L. plantarum* T9 stain incubated in 5% NaCl, the genes encoding the Na^+/H^+ antiporters (*D7Y66_07525*, *D7Y66_13435*) and co-chaperone GroES and GroEL (*D7Y66_03635*, *D7Y66_03640*) showed higher transcriptional levels (changes ≥ 1.0-fold), implying increased antiporting of Na^+/H^+ in *L. plantarum* T9 cells. Comparison of the transcriptional profiles revealed differentially expressed genes that were synchronously elicited from both *L. plantarum* D31 and T9 strains in the salt stress. Nevertheless, opposite transcriptional patterns were also observed in the two strains. For instance, the genes encoding the GroES and GroEL (*D7Y65_06915*, *D7Y65_06920*) were notably downregulated in *L. plantarum* D31 (0.14–0.33 fold, $p < 0.05$). Additionally, Esteban-Torres et al. (2014) have reported a cold-active and salt-tolerant esterase from *L. plantarum*. In this study, the gene (*lp_3505*) encoding the esterase was also examined in *L. plantarum* D31 and T9 strains by the qRT-PCR assay. The resulting data showed that expression of the esterase gene was slightly reduced in *L. plantarum* D31 (0.32 fold, $p < 0.05$) and T9 (0.47 fold, $p < 0.05$) strains, respectively. These results suggested possible strain-specific regulatory mechanisms of *L. plantarum* in the salt stress.

Overall, both *L. plantarum* D31 and T9 strains were able to withstand high osmotic pressure caused by 5.0% NaCl, and *L. plantarum* D31 even to tolerate 8.0% NaCl. Our genomic data, coupled with the previous studies, revealed a complex molecular regulatory network responding to the salt stress in *L. plantarum* (Fig. 7). The salt resistance-associated genes identified in *L. plantarum* D31 and *L. plantarum* T9 genomes fall into at least four distinct categories. One of these is involved in the recovery of intracellular ion balance, e.g., the Na^+/H^+ reverse transport and K^+ transport systems. The genes encoding nitrate/sulfonate/bicarbonate ABC transporters and proline synthesis fall into the second category, which are likely essential for absorption or synthesis of compatible solutes. The third category may contain the genes involved in the regulation of intracellular metabolism balance, e.g., encoding transcriptional factors of GntR, TetR, Crp/Fnr, and LysR families, and a number of response regulators, particularly for the modulation of cell membrane composition changes. The genes involved in the stress response, e.g., encoding one-component regulatory systems DnaK-DnaJ and GroES-GroEL, may go into the fourth category (Fig. 7). The data in this study allowed us to better understand molecular coping strategies for the salt tolerance of *L. plantarum*. The bacterium with novel functional properties is of interest to both academic institution and food industry. Both *L. plantarum* D31 and T9 strains showing high level of salt tolerance are promising components for traditional food fermentations.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s13213-020-01551-2.

Additional file 1: Table S1. The genome features of the 50 *L. plantarum* strains analyzed in this study.

Additional file 2: Table S2. The genes classified into 24 COG functional categories in *L. plantarum* D31 and T9 genomes.

Additional file 3: Table S3. Regulators related to the salt stress in *L. plantarum* D31 and T9 genomes.

Additional file 4: Figure S1. Agarose (0.7%) gel electrophoresis analysis of genomic DNA extracted from *L. plantarum* D31 and T9 strains. (A): Lane 1: DNA molecular Maker: λ/HindIII DNA Marker (Tiangen Biotech Co., Ltd., Beijing, China). Lane 2 and 3: genomic DNA samples extracted from *L. plantarum* D31 and T9 strains, respectively. Electrophoresis was performed at 120 voltage for about 30 min in 1 × TAE buffer (Shanghai Sangon Biological Engineering Technology and Services Co., Ltd., Shanghai, China). (B): The marker ladder photo provided by the manufacturer.

Additional file 5: Figure S2. Agarose (1%) gel electrophoresis analysis of total RNA extracted from *L. plantarum* D31 and T9 strains. Lane 1 and 3: *L. plantarum* D31 and T9 strains grown in MRS medium, respectively. Lane 2 and 4: *L. plantarum* D31 and T9 strains grown in MRS medium supplemented with 8%, and 5% NaCl, respectively.

Additional file 6: Figure S3. Amplification plots of qRT-PCR products derived from the sixteen representative salt resistance-associated genes of *L. plantarum* D31 (A) and T9 (B) strains. The qRT-PCR assay was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) (See Materials and methods). *L. plantarum* D31 and T9 strains grown in MRS medium supplemented with 8%, and 5% NaCl, respectively.

Authors' contributions

WY, LY, ZS, LX, and LC participated in the design and or discussion of the study. WY, and LY carried out the major experiments and analyses. WY, and LC wrote the manuscript. All authors read and approved the final manuscript.

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Ethics approval and consent to participate

This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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