Physiological and transcriptional responses and cross protection of *Lactobacillus plantarum* ZDY2013 under acid stress

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ABSTRACT

Acid tolerance responses (ATR) in *Lactobacillus plantarum* ZDY2013 were investigated at physiological and molecular levels. A comparison of composition of cell membrane fatty acids (CMFA) between acid-challenged and unchallenged cells showed that acid adaptation evoked a significantly higher percentage of saturated fatty acids and cyclopropane fatty acids in acid-challenged than in unchallenged cells. In addition, reverse transcription-quantitative PCR analysis in acid-adapted cells at different pH values (ranging from 3.0 to 4.0) indicated that several genes were differently regulated, including those related to proton pumps, amino acid metabolism, sugar metabolism, and class I and class III stress response pathways. Expression of genes involved in fatty acid synthesis and production of alkali was significantly upregulated. Upon exposure to pH 4.5 for 2 h, a higher survival rate (higher viable cell count) of *Lactobacillus plantarum* ZDY2013 was achieved following an additional challenge to 40 mM hydrogen peroxide for 60 min, but no difference in survival rate of cells was found with further challenge to heat, ethanol, or salt. Therefore, we concluded that the physiological and metabolic changes of acid-treated cells of *Lactobacillus plantarum* ZDY2013 help the cells resist damage caused by acid, and further initiated global response signals to bring the whole cell into a state of defense to other stress factors, especially hydrogen peroxide.

Key words: acid tolerance response, *Lactobacillus plantarum*, physiological and transcriptional level, cross-protection

INTRODUCTION

Lactic acid bacteria (LAB) have traditionally been recognized as probiotics and are used in commercial

products for their health-promoting and nutritional benefits. However, LAB encounter various stress conditions during industrial processes and in the gastrointestinal tract (Mills et al., 2011), including high temperature, ethanol (van Bokhorst-van de Veen et al., 2011), hydrochloric acid, organic acids (lactic acid and acetic acid) (Broadbent et al., 2010; Mols et al., 2010), bile salts (Koskenniemi et al., 2011; Alcántara and Zuniga, 2012), gastric juices, and digestive enzymes (Bove et al., 2013). Among these challenges, acid stress is an inevitable hurdle for the survival of LAB. Several studies have confirmed that administration of high populations of LAB or probiotics provide beneficial effects (Mills et al., 2011; Bove et al., 2013); hence, acid resistance of an organism is a vital physiological attribute for its survival in the gut. Therefore, analysis of the acid tolerance response (ATR) would be helpful for understanding the mechanisms of survival in low pH and other stressful environments.

The possible mechanisms of ATR include proton pumps, production of an alkaline substance, changes in cell membrane, and protection or repair of macromolecules (Cotter and Hill, 2003; Wu et al., 2013). Studies in *Lactobacillus casei* showed that the content of cytoplasmic membrane fatty acid (CMFA) was altered to increase the proportion of C18:1 and cyclopropane fatty acid (C19:0) in response to acid challenge (Broadbent et al., 2010). The H⁺-ATPase pathway, glutamic acid decarboxylation, and production of alkaline substances (through the arginine deiminase system) are the main systems involved in pH homeostasis in LAB (Cotter and Hill, 2003). Genes and proteins involved in pH homeostasis and cell protection or repair play a key role in acid adaptation (Mols et al., 2010; Mols and Abee, 2011). O'Sullivan and Condon (1999) reported that a high level of H⁺-ATPase was associated with strong acid tolerance in *Lactococcus lactis* at sub-lethal levels of acid. Research on *Lactococcus reuteri* 100-23 showed that putative glutamate decarboxylase, which converts glutamate to γ -aminobutyric acid, contributed to its acid resistance (Su et al., 2011). Arginine deiminase and glutamate decarboxylase were reported to confer

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resistance of *Lc. reuteri* 100-23 to lactate (Teixeira et al., 2014). Phosphofructokinase (**Pfk**) and pyruvate kinase (**Pyk**) provide cells with strong ability to control glycolytic flux, resulting in ATP production, thus providing more energy for cells to pump protons under acidic conditions (Koebmann et al., 2005; Viana et al., 2005; Wu et al., 2012a). However, information on the mechanisms for *Lactobacillus plantarum* to modulate ATR and CMFA content and the cross-protective effect on other stresses are not fully understood.

Lactobacillus plantarum ZDY2013, isolated and confirmed in our previous work, is one of the dominant strains in fermented acid bean from a village in the western part of Fujian Province in China. We have demonstrated that the organism exhibits high acid stress resistance (to HCl and gastric juice) and possesses antibacterial and gut microbiota modulation properties (Huang et al., 2015).

In this study, we analyzed physiological changes in the cells of *Lb. plantarum* caused by exposure to a low pH environment and examined changes in the cell membrane lipid composition using GC-MS. Reverse transcription quantitative PCR (**RT-qPCR**) was used to determine the changes in transcription levels of genes involved in CMFA synthesis, cellular metabolism, energy production, and class I and class III stress response pathways. Furthermore, we examined the cross-protective effect of ATR on other environment stressors, including high temperature, ethanol, salt, and hydrogen peroxide.

MATERIALS AND METHODS

Strain and Growth Conditions

Lactobacillus plantarum ZDY2013 was isolated as previously reported (Huang et al., 2015). A single colony of *Lb. plantarum* ZDY2013 was inoculated in 5 mL of de Man, Rogosa, and Sharpe (**MRS**) broth at pH 6.5, followed by an overnight incubation at 37°C. Cells at log phase (10^7 cfu/mL) were transferred to a 0.5-L triangle bottle and grown overnight (24 h); early stationary phase cells were used to prepare cell suspensions. A typical *Lactobacillus* spp., *Lactobacillus plantarum* ATCC 8014, was used as a comparison.

Measurement of Intracellular pH and ATP

The cell suspension was adjusted the pH to 6.5, 4.5, and 3.0 using HCl (1 M), and incubated at 37°C for 1 h. Viable cells were enumerated after acid challenge. Intracellular pH (**pHi**) was measured by using the fluorescence method developed by Breeuwer et al. (1996), using 5 (and 6-)-carboxyfluorescein succinimidyl ester

as the fluorescent probe. Loading of cells with 5 (and 6-)-carboxyfluorescein succinimidyl ester, determination of pHi, and calibration of pHi all followed the procedures described previously (Breeuwer et al., 1996).

The ATP assay was carried out by using an ATP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's protocol. The final ATP concentration was expressed in nanomoles per milligram of protein. All ATP concentration values were the means of at least 3 independent extractions.

Analysis of CMFA Composition Using GC-MS

Lactobacillus plantarum ZDY2013 was grown in MRS broth for 24 h at 37°C, and 1 M HCl was used to adjust the pH to 6. Then, the cells were exposed to pH 3.5 and incubated for 2 h, followed by CMFA extraction from the cells. All solvents used in preparation of FAME were HPLC grade, and all glassware was washed with distilled water in an ultrasonic cleaner before use. The FAME of CMFA were prepared as described previously (Sasser, 1990; Wu et al., 2012b) with some modifications. Briefly, cells were collected by centrifugation ($5,500 \times g$ for 5 min); then, cell pellets were washed with cold PBS buffer twice, and 1 mL of NaOH in a methanol-distilled water solution (3:10:10 wt/vol/vol) was added. The suspension was heated at 100°C for 30 min, and then cooled to room temperature (24°C), and 2 mL of methanol:HCl:water (22:13:13 vol/vol/vol) solution was added for methylation and the suspension heated at 80°C for 10 min. After rapid cooling in an ice bath to 0°C, FAME were extracted using 1.25 mL of methyl *tert*-butyl ether:hexane (1:1 vol/vol) for 10 min and washed with 3 mL of 0.33 M NaOH. The upper layer was transferred into a 2-mL vial using a Pasteur pipette, and evaporated to about 1 mL under a flow of nitrogen. For GC-MS analysis, 1 μ L of sample was injected into a gas chromatograph (6890N, Agilent, Palo Alto, CA); the carrier gas was hydrogen (1.0 mL/min). Running conditions were injector, 230°C; ionization detector, 250°C; and program temperature ramping from 120°C to 230°C at 6°C/min. The relative contents of FAME were determined by area normalization.

RNA Extraction and cDNA Synthesis

For transcriptional analysis, cells were centrifuged and pellets suspended in 10 mL of MRS, followed by adjustment of the pH to 6.5 (as unstressed control), 4.0, 3.5, and 3.0. Both unstressed and acid-stressed bacterial suspensions were incubated at 37°C for 30 min and then prepared for RNA extraction.

Total RNA was extracted with Transzol reagent (TransGen Biotech, Beijing, China). Integrity and concentration of RNA were determined by agarose gel electrophoresis and spectrophotometer (NanoDrop 1000, Thermo Scientific, Waltham, MA). An aliquot (500 ng) of total RNA was used to synthesize cDNA by using a reverse transcription kit (Takara, Dalian, China) after genomic DNA was removed.

Analysis of Gene Expression by qPCR

To assess the transcriptional level of genes associated with the stress response, qPCR was performed on an ABI 7900HT fast real-time PCR system (Applied Biosystems, Waltham, MA). Two microliters of 10-fold diluted cDNA was added to 15 μ L of a real-time PCR mix containing 10 μ L of SYBR Primer EX *Taq*II (Takara), 0.4 μ L of ROX reference dye (50 $\times$; Takara), 0.2 μ M of each primer (Supplementary Table S1; <http://dx.doi.org/10.3168/jds.2015-9993>), and 6 μ L of Milli-Q H₂O (Millipore Corp., Billerica, MA). Thermal cycling started at 95°C for 1 min followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. The relative transcriptional level was analyzed through the $2^{-\Delta\Delta C_t}$ (delta cycle threshold) method based on qPCR data according to Feng et al. (2010). Untreated bacterial cell suspension served as a calibrator condition, and expression of the lactate dehydrogenase D gene (*ldhD*) was monitored to normalize the expression of target genes (Desroche et al., 2005; Fiocco et al., 2007; Bove et al., 2013). Melting curve analyses were performed to confirm the specificity of reaction.

Cross-Protection Studies

Gene expression responses, especially class I and class III stress regulation members, in *Lb. plantarum* are differentially regulated in various different stress conditions, including ethanol, oxidative, solvent, and cold and heat stress according to van Bokhorst-van de Veen et al. (2013). To determine the cross-protection effect, the tolerance of untreated (unstressed) and pH 4.5 acid-stressed cells were compared under different environmental stress challenges. *Lactobacillus plantarum* ZDY2013 grown in MRS broth at pH 6.5 was used as the control (unstressed condition). An aliquot (5 mL) was acid stressed for 2 h at pH 4.5 and then washed in PBS before exposure to other stressors. For all stress tolerance assays, aliquots were serially diluted immediately after stress exposure, and then immediately plated on MRS agar and incubated at 37°C for 2 d before enumeration of colony-forming units.

To quantify survival of *Lb. plantarum* ZDY2013 after oxidative stress, ZDY2013 cells before and after acid

response were suspended in PBS with 40 mM hydrogen peroxide. Cells were collected every 10 min for enumeration. Cross-protection with ethanol stress was determined after the cells were suspended in PBS with 8% (vol/vol) ethanol. Subsequently, samples were collected at every 2 h in triplicate, followed by enumeration of viable cells. Similarly, for osmotic pressure and salt stress, cells were suspended in PBS with 3% NaCl. The heat resistance of ZDY2013 cells treated in pH 6.0 or 4.5 was assessed after suspending the cells in PBS and exposing them to 65°C for 30 min or 100°C for 10 min.

Statistical Analysis

All results were expressed as means $\pm$ standard deviations. Statistical analysis was performed using one-way ANOVA procedure of SPSS 13.0 software (SPSS Inc./IBM Corp., Chicago, IL). All experiments were performed in triplicate and all the analyses were carried out in duplicate.

RESULTS

Intracellular pH and ATP

The population decrease following acid challenge was related to the decrease in pH_i. As shown in Figure 1A, *Lb. plantarum* ZDY2013 exhibited a higher survival rate during acid challenge than did *Lb. plantarum* ATCC 8014. Furthermore, at pH 6.5, there was no obvious difference in pH_i and ATP levels between *Lb. plantarum* ZDY2013 and *Lb. plantarum* ATCC8014 (Figure 1B and 1C). However, a higher pH_i and ATP level were detected in *Lb. plantarum* ZDY2013 at both pH 4.5 and 3.0 compared with that in *Lb. plantarum* ATCC 8014. *Lactobacillus plantarum* ZDY2013 (with high acid resistance) had a relatively higher pH_i. The high ATP levels further suggested that *Lb. plantarum* ZDY2013 might effectively prevent the rapid decline of pH_i during acid challenge.

Lipid Composition

Cell membrane regulating substances entering or exiting cells play an important role in cellular growth, metabolism, energy transduction, and maintenance of a constant intracellular environment. The membrane fluidity of bacteria is decreased by changing the fatty acid composition to defend proton influx for the maintenance of pH homeostasis (Mykytczuk et al., 2007; Zhang and Rock, 2008). In this study, the main CMFA in *Lb. plantarum* ZDY2013, including C14:0, C16:0, C16:1, C18:0, C18:1, C18:2, and C19:0, made up over

Figure 1. Changes in (A) survival rate, (B) intracellular pH (pH_i), and (C) intracellular ATP concentration during acid stress. Cells resuspended at pH 6.5 were exposed at pH 4.5 or 3.0 for 1 h. White bars represent *Lactobacillus plantarum* ATCC 8014, and black bars represent *Lactobacillus plantarum* ZDY2013. Error bars indicate standard deviations (n = 3). Statistically significant differences ($P < 0.05$) were determined by Student's *t*-test and are indicated with an asterisk.

Table 1. Effect of acid challenge on the cell membrane fatty acid (CMFA) composition (% of FA in total CMFA; mean $\pm$ SD) of *Lactobacillus plantarum* ZDY2013

Fatty acid	Treatment	
	Control	Acid treated
C12:0	0.221 $\pm$ 0.610	0.381 $\pm$ 0.089
C14:0	3.228 $\pm$ 0.467	4.239 $\pm$ 0.860
C16:0	28.414 $\pm$ 2.602	34.285 $\pm$ 1.509*
C18:0	1.298 $\pm$ 0.219	0.972 $\pm$ 0.122
C16:1	4.382 $\pm$ 0.303	3.437 $\pm$ 0.203
C18:1	29.872 $\pm$ 1.933	28.155 $\pm$ 5.603
C18:2	22.518 $\pm$ 7.565	7.087 $\pm$ 2.527*
C19:0(9c)	10.068 $\pm$ 2.430	21.443 $\pm$ 3.717*
SFA (%)	30.160 $\pm$ 2.629	38.254 $\pm$ 2.734
Unsaturated FA (%)	69.840 $\pm$ 2.629	61.746 $\pm$ 2.734
SFA:unsaturated FA	0.432 $\pm$ 0.059	0.663 $\pm$ 0.012**

* $P < 0.05$, ** $P < 0.01$, compared with unstressed condition.

96% of the total CMFA content (Table 1). After acid treatment (pH 3.0 for 2 h), the ratio of SFA to unsaturated FA in treated cells was as high as 0.66, which was significantly higher than that for the control cells (0.43; $P < 0.01$). The percentages of SFA (e.g., C14:0, C16:0) and cyclopropane (C19:0 *cis*-9) in acid-treated cells were higher than that of the control, whereas PUFA (C18:2) exhibited a significant decrease ($P < 0.05$). *Lactobacillus plantarum* ZDY2013 actively adjusted membrane fluidity by changing the lipid composition, indicating that the cell envelope was adapted to cope with the extreme acid condition.

Transcriptional Analysis in Acid Response

To further explore global changes in genes at the transcriptional level during acid stress, we carried out a comprehensive RT-qPCR analysis. A set of *Lb. plantarum* genes potentially involved in survival and stress tolerance was analyzed. The gene transcripts that were up- or downregulated belonged mainly to the following functional categories: (1) CMFA synthesis; (2) cellular metabolism and energy production, including amino acid and sugar metabolism, genes connected to proton pumps, and production of alkali; and (3) class I and class III stress response pathways, including transcriptional controller, posttranslational modification, protein turnover, and chaperones (van Bokhorst-van de Veen et al., 2011, 2013; Wu et al., 2012a; Bove et al., 2013).

Membrane Lipid Synthesis

We observed an obvious difference in gene expression of CMFA synthesis between pH 6.5 (control) and those at pH 4.0, 3.5, 3.0 (Figure 2A). Transcription

levels of acyl carrier proteins (*acp*) and fatty acid/phospholipid synthesis protein (*plsX*) were upregulated 3.46- and 1.56-fold, respectively, after acid treatment at pH 3.0 for 2 h. In addition, genes encoding fatty acid elongation proteins (*fabF*, *fabZ*, *fabG*) were activated at pH 3.0 and were increased 6.13-, 5.83-, and 5.02-fold, respectively. Remarkably, cyclopropane fatty-acyl-phospholipid synthase (*cfa1*) was upregulated 1.54-fold, whereas its paralogue, *cfa2*, was repressed. These results indicate that *Lb. plantarum* ZDY2013 adapted its cell membrane structure by adjusting the transcription levels of genes involved in CMFA synthesis in response to acid stress.

Cellular Metabolism and Energy Production

Enzymes participating in glycolysis and ATP production may support an increase in H^+ expulsion under acid conditions. In this study, transcription levels of genes related to sugar catabolism, including phosphoglycerate kinase (*pgk*), phosphocarrier protein (*ptsh*), 6-phosphofructokinase (*pfk*), and pyruvate kinase (*pyk*), were enhanced 1.08-, 1.92-, 2.24-, and 1.33-fold, respectively, during acid stress compared with that of the control (Figure 2B). These enzymes catalyze the conversion of fructose 6-phosphate and phosphoenolpyruvic acid into fructose 1,6-diphosphate and pyruvic acid, respectively. In addition, 3 genes (*atpA*, *atpD*, and *gspA*) related to cellular metabolism and energy production were differentially expressed in acid-stressed and unstressed cells. In response to acid stress, genes involved in ATP synthase (*atpA* and *atpD*) and glycerol-3-phosphate dehydrogenase (*gspA*) were upregulated 1.36-, 2.27-, and 2.04-fold, respectively, compared with those of unstressed cells (Figure 2C).

Conversion of aspartate into arginine can improve arginine catabolism and result in the production of ammonia, which can increase the intracellular pH, concomitantly producing ATP. In this study, 4 genes involved in aspartate and arginine pathways were examined, including aspartate-ammonia ligase (*asnA*), argininosuccinate synthase (*argG*), ornithine carbamoyltransferase (*argF*), and arginine catabolic regulator (*argR*). It is worth mentioning that gene *asnA*, which catalyzes the conversion of aspartate into asparagine, was downregulated, whereas 3 other genes, *argG*, *argF*, and *argR*, which are involved in arginine synthesis and catabolism, were significantly upregulated. In particular, the transcription levels of *argG* and *argF* increased by 5.26- and 17.90-fold, respectively, at pH 3.0 (Figure 2D), compared with those of the unstressed cells. Consequently, the flux from aspartate to arginine increased but the flux to asparagine decreased. Another upregulated pathway in the acid-stressed cells was the conver-

sion of glutamate into γ -aminobutyrate by glutamate decarboxylases.

Glutamate decarboxylases can combine a glutamate with an intracellular proton and exchange the glutamate for the more alkaline γ -aminobutyrate, which can neutralize the extracellular pH slightly. Furthermore, consumption of a proton resulted in an increase in the intracellular pH. The glutamate decarboxylase gene (*gadB*) was found to be upregulated 1.5-fold at pH 3.0 when cells were stressed for 30 min (Figure 2C). The glutamate dehydrogenase gene (*gdh*), which can catalyze α -ketoglutaric acid to form glutamate, also increased 2.3-fold. The increase in the glutamate synthesis flux could result in an increase in the substrate concentration of glutamate decarboxylases.

Induction of Class I and Class III Stress Response Pathways

Stress response transcriptional regulators HrcA and CtsR, which are associated with tolerance to one or more environmental stresses, as well as the genes under their control were differentially expressed under acid stress (Figure 2E). Transcriptions of *hrcA* and 2 other genes that are predicted to be regulated or at least partially controlled by *hrcA*, *dnaK*, and *groEL* (encoding chaperones), were significantly upregulated after acid stress for 30 min. Transcription of *ctsR* was significantly repressed during acid stress, whereas genes predicted to be repressed by *ctsR*, including *clpB*, *clpP*, and *clpE* (encoding proteases), were upregulated after acid stress. Other genes associated with tolerance to one or more environmental stresses were also differentially regulated after 30 min of acid stress, including proteolytic enzyme (*ftsh*, 2.0-fold), and heat response genes *hsp1* and *hsp3* (small heat shock protein, 1.8- and 3.6-fold, respectively; Figure 2E).

Cross-Protective Effect of Acid-Induced *Lb. plantarum* ZDY2013 Cells

Because class I and class III stress response pathways were activated in *Lb. plantarum* ZDY2013 during growth in acid stress, we examined whether this strain could withstand other chemical or environmental stresses after exposure to acid stress. To examine the cross-protection of *Lb. plantarum* ZDY2013, cultures kept at pH 4.5 for 2 h were resuspended in PBS with 40 mM hydrogen peroxide or 8% ethanol, or were exposed to high temperatures (63°C and 100°C), or to 5% NaCl. Among these stress conditions, only the resistance to hydrogen peroxide was enhanced significantly ($P < 0.05$) and no difference was found in the survival of acid-stressed cells when exposed to other stressors.

Figure 2. Transcriptional levels of acid stress-related genes in *Lactobacillus plantarum* ZDY2013 after acid stress for 30 min. The results are exhibited as fold changes of mRNA levels compared with control cells (unstressed, pH 6.5) and normalized using lactate dehydrogenase (*ldhD*) as internal control. Data are the means and standard deviations of 3 independent experiments. Genes related to (A) cell membrane fatty acid synthesis, (B) sugar catabolism, (C) proton pumps (including H-ATPase pathway and GAD pathway), (D) production of alkali (arginine deiminase pathway), and (E) class I and class III stress response pathways. Error bars indicate standard deviations ($n = 3$). Statistically significant differences ($P < 0.05$) are indicated with an asterisk.

Figure 3. Cross protection against hydrogen peroxide of *Lactobacillus plantarum* ZDY2013 after adjusting the pH of de Man, Rogosa, and Sharpe broth to pH 4.5 (gray bars) or control (pH 6.5, white bars) for 2 h and subjected to 40 mM hydrogen peroxide. Error bars indicate standard deviations ($n = 3$). Statistically significant differences ($P < 0.05$) are indicated with an asterisk.

Lactobacillus plantarum ZDY2013 treated in MRS at pH 4.5 for 2 h maintained greater survival in PBS containing 40 mM hydrogen peroxide during 60 min compared with the control (Figure 3). The viable cell counts of ZDY2013 in the control (pH 6.5) decreased rapidly when exposed to hydrogen peroxide at 40 mM. However, cell counts of *Lb. plantarum* ZDY2013 at pH 4.5 were stable for the first 10 min when exposed to hydrogen peroxide, but subsequently decreased from 20 min and on.

DISCUSSION

Lactobacillus species, which are able to grow and survive under suboptimal conditions during food fermentation and gastrointestinal tract digestion, have attracted a lot of interest for their stress response mechanisms. In this study, we unraveled the adaptation strategy of *Lb. plantarum* ZDY2013 that enables it to grow in medium at pH 3.5 (Huang et al., 2015). This strain was shown to adapt by modulating basic metabolic pathways and cell membrane composition and by inducing stress response pathways. Transcriptional responses were elicited within 30 min of exposure to MRS medium at pH 4.0, 3.5, and 3.0. These adaptations resulted in cross-protection against hydrogen peroxide stress but not against other stressors.

A highly acidic environment results in the reduction of cytoplasmic pH (pH_i), which induces loss of activity of

relevant acid-sensitive glycolytic enzymes and structural damage to macromolecules such as DNA and proteins (Cotter and Hill, 2003). As the first barrier between the external environment and the intracellular medium, the cell membrane is the primary defense against damage induced by environmental stress (Streit et al., 2008; Wu et al., 2012b). Moreover, bacteria decrease membrane fluidity by changing the fatty acid species and composition in defense of proton influx (Mykytczuk et al., 2007; Zhang and Rock, 2008). Analysis by GC-MS of the CMFA composition of *Lb. plantarum* ZDY2013 demonstrated that the level of C18:2 in the cell membrane of *L. plantarum* ZDY2013 was reduced and the ratio of total SFA to unsaturated FA increased in acid-treated cells compared with unstressed cells. Changes in fatty acid levels were accompanied by changes in the expression of the corresponding genes; that is, synthesis of enzymes (as shown in Figure 2A). Therefore, the changes in lipid composition as monitored by GC-MS and regulation of transcription of genes related to CMFA synthesis indicated that cell membrane regulation played an important role in the ATR process of *Lb. plantarum* ZDY2013.

The core response to cope with the acid environment included intracellular ATP level. Bacteria modulate various basic metabolic pathways to enhance ATP production, which can help to pump protons out of cells (Broadbent et al., 2010). Previous studies reported that expression of phosphofructokinase (*pfk*) and pyruvate kinase (*pyk*; glycolytic enzymes that severely affect a cell's ability to produce ATP) provided cells the potential to control glycolytic flux (Koeblmann et al., 2005; Viana et al., 2005). Similarly, in our study, acid-treated cells of ZDY2013 exhibited higher transcript levels of *pfk* and *pyk* than did the unstressed cells. Wu et al. (2012a) reported that the mutant of *Lb. casei* Zhang with enhanced tolerance to lactic acid exhibited higher expression of *pfk* and *pyk* during acid stress. Therefore, the response of ZDY2013 enhanced sugar metabolism flux, which leads to an increase in ATP production to provide more energy to pump protons out of cells (Jin et al., 2012).

Transcriptional analyses of ZDY2013 verified that the genes *atpA* and *atpC*, which encode H^+ -ATPase, were upregulated at pH 4.0 or at lower pH. Proton-translocating ATPase (H^+ -ATPase) expels protons at the expense of consumption of ATP, which contributes to the expulsion of protons and consequently confers resistance to stress (McDonald et al., 1990; Hutkins and Nannen, 1993). Cytoplasmic pH (pH_i) is reported to correlate with the cellular level of H^+ -ATPase (O'Sullivan and Condon, 1999).

Production of alkali was another core response of *Lb. plantarum* to acid stress. Transcriptional analyses

suggest that *argG* and *argF*, core genes involved in the arginine deiminase system, were dramatically upregulated under acid stress in the current study. The enhancement of flux from aspartate to arginine could lead to the production of ammonia and 1 mol of ATP per mol of arginine consumed (Wu et al., 2012a). Overall, ATP supplement system and pH neutralization play an important role in the physiological regulation of *Lb. plantarum* ZDY2013 under acid stress.

Although we detected some overlap of gene transcriptional responses between one environment stress and another, adaptation of *Lb. plantarum* to acid stress cross-protected this organism only against hydrogen peroxide. Similarly, van Bokhorst-van de Veen et al. (2011) noted that the induction of CtsR class III stress responses toward ethanol exposure provided cross protection to *Lactobacillus plantarum* WCFS1 against thermal stress but not against oxidative or high-salt stress. Generally, different cells initiated global response signals to bring the whole cell into a state of response to the stress, and thus exhibited resistance against other stressful environments; different cells presented different types of cross protection. For example, the induction of alkaline shock response enhances resistance of *Enterococcus faecalis* ATCC 19433 to bile salt (Flahaut et al., 1997).

Generally, there is some overlap of stress response for *Lb. plantarum* to cope with different environments. van Bokhorst-van de Veen et al. (2013) reported that the CtsR and HrcA regulatory networks were invoked in different stress responses of *Lb. plantarum* and illustrated that stress responses adapted with a complex nature. Similarly, a set of genes involved in tolerance to one or more environmental stresses was induced during the acid stress response in ZDY2013 (Figure 2E), including genes encoding regulatory proteins (driving genetic responses; i.e., *ctsR* and *ftsH*), chaperones (assisting in folding of misfolded proteins; i.e., *dnaK* and *groEL*), and proteases (degrading irreversibly damaged proteins; i.e., *clpP*), which have been identified in various stress conditions (Wall et al., 2007; Seydlová et al., 2012; Bove et al., 2013; van Bokhorst-van de Veen et al., 2013). The *groEL* and *dnaK* genes are class I heat shock genes controlled by the HrcA repressor (Schumann, 2003; Spano and Massa, 2006), whereas *ftsH* and *clpP* genes belong to the CtsR regulon of *Lb. plantarum* (Wall et al., 2007; Fiocco et al., 2009; Bove et al., 2012).

Supported by the physiological changes identified by GS-MS and transcriptional changes identified by RT-qPCR, this study demonstrates that prior exposure to acid stress not only induces an ATR but also induces protection to stress from hydrogen peroxide.

CONCLUSIONS

In this study, *Lb. plantarum* ZDY2013 exhibited a complex adaptation strategy to deal with acid stress by changing fatty acid biosynthesis, regulating transcription level of genes associated with glycolysis and proton pumps, producing alkaline substances, and improving translational accuracy and protein folding, as well as modulating transcriptional regulation networks (class I and III stress response pathways). However, the physiological and molecular responses did not provide cross-protection against heat stress, ethanol, or bile salt stress, only against hydrogen peroxide stress.

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