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Systemic understanding of *Lactococcus lactis* response to acid stress using transcriptomics approaches

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Abstract

During fermentation, acid stress caused by the accumulation of acidic metabolites seriously affects the metabolic activity and production capacity of microbial cells. To elucidate the acid stress-tolerance mechanisms of microbial cells, we performed genome mutagenesis combined with high-throughput technologies to screen acid stress-tolerant strains. Mutant strain *Lactococcus lactis* WH101 showed a 16,000-fold higher survival rate than that of the parent strain after 5 h of acid shock at pH 4.0 and maintained higher ATP, NH_4^+, and intracellular pH (pH_i) levels during acid stress. Additionally, comparative transcriptomics analysis revealed enhanced regulation of carbohydrate metabolism and sugar transport to provide additional energy, amino acid metabolism and transport to maintain pH_i homeostasis and ATP generation, and fatty acid metabolism to enhance cellular acid tolerance. Moreover, overexpression of identified components resulted in 12.6- and 12.9-fold higher survival rates after acid shock for 3 h at pH 4.0 in *L. lactis* (ArcB) and *L. lactis* (MalQ) compared to the control strain, respectively. These findings provide valuable insight into the acid stress-response mechanisms of *L. lactis* and promote the further development of robust industrial strains.

Keywords *Lactococcus lactis* · High-throughput screening · Acid stress · Transcriptomics · Metabolic engineering

Introduction

In the industrial fermentation process, the production of organic acids, amino acids, and other acidic products can seriously affect cell growth. Improving the acid tolerance of bacteria is beneficial to extend the fermentation process and enhance cell viability, both of which are important for the efficient production of acid metabolites [9]. *Lactococcus lactis* is widely employed in the dairy industry to produce various fermented foods. The optimal pH for growth of *L. lactis* has been found to occur in a range of 6.3–6.9. During growth, the pH of media generally reduces to approximately 4.5 due to the accumulation of acidic metabolites [17]. Moreover, *L. lactis* is frequently confronted with various environmental stresses, especially acid stress [22]. The activities of essential enzymes will be inhibited, and thus the metabolic activity and production efficiency of microbial cells will be affected [2]. Therefore, improving acid stress tolerance may contribute to the industrial application of *L. lactis*.

Recently, the advent of systems biology and high-throughput technologies has allowed the enhanced understanding of acid-tolerance mechanisms at the systemic level. Omics technologies are effective at investigating the mechanisms associated with microbial cell response to acid stress [26]. A previous study used comparative genomics and transcriptomics analysis to understand the propionic acid-response mechanisms of *Propionibacterium acidipropionici* [8]. Moreover, a proteomics approach combined

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with transcription analysis revealed several possible acid-tolerance mechanisms in *Lactobacillus delbrueckii* subsp. *bulgaricus* CAUH1 and identified a novel acid tolerance-related transcriptional regulator (Ldb0677) [24].

Based on the identification of anti-acid components, a series of metabolic engineering strategies were developed to improve the acid tolerance of microbial cells. Co-expression of noncoding small RNAs DsrA, RprA, and ArcZ increased acid tolerance in *Escherichia coli* and protected cells against carboxylic acid and oxidative stress [6]. Heterologous expression of small heat-shock protein Lo18 improved the stress tolerance of *L. lactis* in milk medium [19]. Moreover, various types of anti-acid components were identified and engineered to improve the acid tolerance of bacteria [1, 16]. Nevertheless, the regulatory network associated with acid tolerance is complex in *L. lactis,* and some acid-response mechanisms remain unclear. Therefore, investigating acid stress-tolerance mechanisms in *L. lactis* NZ9000 and constructing robust industrial strains are topics of considerable importance.

In this study, three acid-tolerance mutant strains were screened using mutagenesis combined with high-throughput screening strategies. To further reveal the acid-tolerance mechanisms of *L. lactis*, comparative transcriptome analysis was performed with mutant strain *L. lactis* WH101 and parent strain *L. lactis* NZ9000. Furthermore, we constructed two recombinant strains based on the identified anti-acid components and evaluated their acid stress tolerance. This study describes a new strategy for performing mutagenesis-based transcriptome analysis to promote the construction of acid stress-tolerant industrial strains.

Materials and methods

Bacterial strains, plasmids, and culture conditions

All strains and plasmids used in this study are listed in Table S1. *L. lactis* NZ9000 was the ancestral strain of the three mutant populations. *L. lactis* cells were cultured in GM17 medium (M17 broth supplied with 0.5% glucose) at 30 °C without shaking (Oxoid M17 broth; Thermo Fisher Scientific, Waltham, MA, USA). *E. coli* MC1061 was used for plasmid preparation and incubated in Luria–Bertani (LB) medium at 37 °C with shaking at 220 rpm. When appropriate, LB and GM17 media were supplemented with 100 μg/ml and 10 μg/ml chloramphenicol, respectively.

Strategies for high-throughput screening of acid-tolerant strains

The high-throughput screening process is shown in Fig. 1a. Two approaches were used for mutagenesis of the parent strain. Cell concentrations at the exponential-growth phase were adjusted to between 10^7 and 10^8 CFU/mL, and ultraviolet (UV) mutagenesis was performed as follows: 4 mL of cell suspension was transferred to a 9-cm plate, which was exposed to UV irradiation for up to 60 s at a distance of 30 cm using a UV lamp at 15 weeks. For chemical [diethyl sulfate (DES)] mutagenesis, 4 mL of cell suspension was treated with 20 μL DES at 30 °C for up to 40 min. Mutated cells were sampled at various times, and the lethality rate of each treatment condition was determined according to the number of surviving colonies.

Mutated cells were transferred to 96-well plates containing GM17 medium (pH 5.0) to an initial optical density at 600 nm (OD_{600}) of 0.1. Mutated cells with higher growth rates compared to that of the wild type were selected and transferred to GM17 agar plates (pH 5.2). Individual colonies were picked and used to inoculate 96-well plates for the second round of screening.

Growth performance and stress-tolerance assays

To assess *L. lactis* growth, cells in the exponential phase (at 4 h) were used to inoculate GM17 media to an initial OD_{600} of 0.1 at pH 7.0, pH 5.0, and pH 4.5 by the addition of lactic acid, respectively. Growth was monitored by determining the OD_{600} value on a spectrophotometer (BioTeK, Winooski, VT, USA). Lactate concentration was measured using a glucose–lactate analyzer (SBA-40C, Biology Institute of Shandong Academy of Sciences, Jinan, China).

To determine *L. lactis* acid tolerance, cells in the exponential phase were harvested and washed twice with 0.85% saline solution and resuspended in an equal volume of modified GM17 medium (adjusted to pH 4.0 with lactic acid). For assessment of other types of stress, cells in the exponential phase were exposed to 15% ethanol, 15% NaCl, and 1 mM H_2O_2, respectively. Cell viability was determined by colony counting after 10 μL of serially diluted cell suspension spotted on GM17 agar plates and incubated at 30 °C for 24 h. Each sample was assayed in triplicate, and colonies containing between 10 CFU and 200 CFU were counted.

Measurement of intracellular ATP concentration, ammonia level, and pH_i concentration

For the measurement of intracellular ATP concentration, cells in the exponential phase were subjected to acid stress (pH 5.0, adjusted with lactic acid) and then sampled at various times. Cellular metabolism was quenched using liquid nitrogen (4 mL of the culture was exposed to liquid nitrogen for 5 min) and centrifuged at 10,000*g* for 10 min at 4 °C, followed by harvesting of the pellets. Intracellular ATP concentration was determined using an ATP assay kit (Beyotime, Shanghai, China) according to the manufacturer instructions.

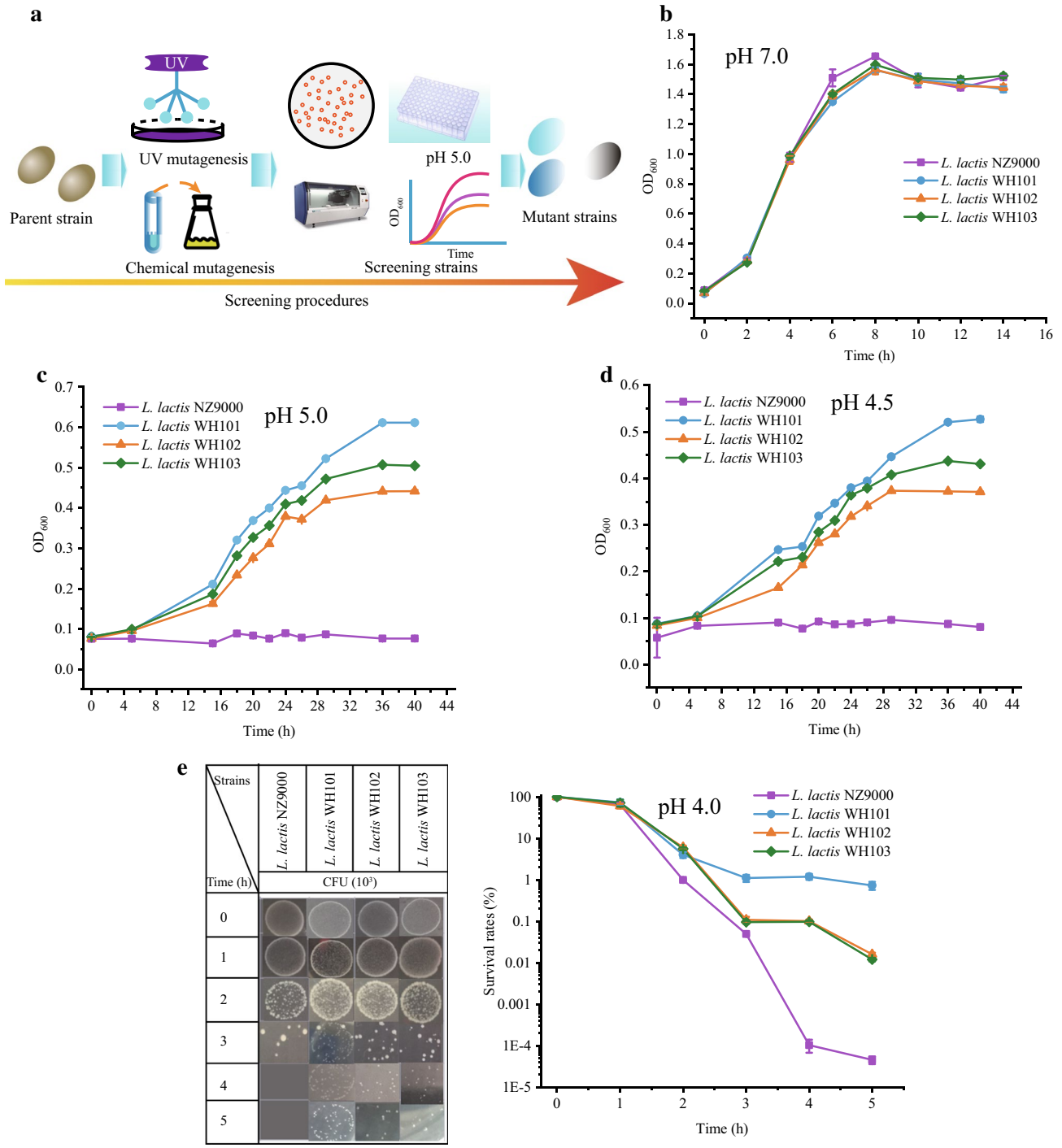

Fig. 1 High-throughput screening of *L. lactis* mutant strains. **a** The high-throughput screening process. Treated cells were transferred to 96-well plates with GM17 medium (pH 5.0) and the OD_{600} was determined. Well-growing cells were selected for transfer to GM17 agar plates (pH 5.2), where colonies were subsequently selected to inoculate 96-well plates for further screening. **b**–**d** The growth phenotypes of the parent and three mutant strains at pH 7.0 (**b**), pH 5.0 (**c**), and pH 4.5 (**d**). **e** The acid stress tolerance of all four strains at pH 4.0 for various times. The pH was adjusted by the addition of lactic acid. Error bars represent the mean ± standard deviation of three replicates

The final ATP concentration was expressed as nanomole per milligram protein.

For the measurement of intracellular ammonia level, harvested cells (pH 5.0) were washed twice with ice-cold 50 mM potassium phosphate buffer (PBS pH 7.4) and resuspended in an equal volume of buffer. Cells were disrupted using a FastPrep-24 Classic instrument (MP Biomedical, Santa Ana, CA, USA) at 4 m/s for 20 s, followed

by six additional rounds of sonication at 2 min intervals on ice. The ammonia concentration was determined using an ammonia assay kit (ScienCell, Carlsbad, CA, USA) according to the manufacturer's instructions. The ammonia concentration was expressed as micrograms per milligram of protein.

For the measurement of pH_i, cells under different acid stress conditions (pH 7.0, 6.0, and 5.0) were harvested at 4 h by centrifugation at 10,000*g* for 5 min, and the pH_i was measured using the fluorescence method described previously [7]. The protein concentration of each sample was determined using the BCA protein assay kit (Tiangen, Beijing, China), with bovine serum albumin used as a standard.

RNA-Seq sample preparation, gene expression analysis, and transcriptome data verification

After reaching the exponential phase, the first aliquot of 20 mL from the culture was harvested (0 h) and used as an unstressed group. Meanwhile, the remaining culture (20 mL) was subjected to acid stress (pH 4.0, adjusted with lactic acid) for 4 h, followed by collection by centrifugation at 8000*g* for 4 min at 4 °C and washing twice with ice-cold 50 mM PBS. The pellets were ground into powder using liquid nitrogen, and total RNA was extracted using the RNAprep pure bacteria kit (Tiangen) according to the manufacturer's instructions. Purified RNA was quantified using a NanoDrop ND-2000 (Thermo Fisher Scientific). RNA samples were stored at − 80 °C until the transcriptome analysis. Samples were sent to BGI (Shenzhen, China) for transcriptome sequencing, which was performed using an Illumina HiSeq 2000 system (Illumina, San Diego, CA, USA).

After quality control, the raw reads were filtered into clean reads and aligned to the reference sequences (accession version: NC_017949.1) using SOAPaligner/SOAP2 [13], and gene expression levels were calculated using the reads per kilobase transcript per million mapped reads method [15]. The significance of differences in gene expression was screened according to a false-discovery rate ≤ 0.001 and fold changes ≥ 2. The Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes, and Genomes (KEGG) enrichment analysis used publicly available data from the Gene Ontology Database (http://www.geneontology.org/) and the KEGG database, respectively. The raw data of transcriptomics obtained in this study were deposited in the NCBI Gene Expression Omnibus under accession number GSE116952.

Transcriptome data verification was conducted with real-time quantitative reverse transcription PCR (qRT-PCR) experiment (Supplementary methods). Primer sequences are listed in Table S2.

Construction of overexpression strains

The *arcB* and *malQ* genes were amplified using *L. lactis* NZ9000 chromosomal DNA as a template, and *Nco*I and *Hind*III restriction sites were simultaneously inserted into the amplified gene fragments. The resulting fragments were digested with *Nco*I and *Hind*III and ligated into plasmid pNZ8148, which was digested with the same restriction enzymes. The ligated products were transformed into *E. coli* MC1061, and positive clones were chosen according to colony PCR, followed by restriction-enzyme digest and sequencing. The recombinant plasmids were named pNZ8148/ArcB and pNZ8148/MalQ, respectively, and subsequently electro-transformed into *L. lactis* NZ9000 [10]. The resulting recombinant strains were named *L. lactis* (ArcB) and *L. lactis* (MalQ), respectively. The empty pNZ8148 plasmid was transformed into *L. lactis* NZ9000 as a control, resulting in *L. lactis* (Vector). All primers used in this study are listed in Table S3.

Results and discussion

High-throughput screening of *L. lactis* mutant libraries

We screened acid-tolerant strains by employing UV and DES mutagenesis, combined with high-throughput screening strategies. With a lethality rate of 85.3%, two DES mutants that showed dramatically increased resistance to acid stress compared to the parent strain were obtained from 20,000 mutant colonies and subsequently named *L. lactis* WH101 and *L. lactis* WH102. Similarly, with a lethality rate of 92.1%, one UV mutant was obtained from 15,000 mutant colonies and named *L. lactis* WH103 (Fig. S1).

To clarify the effects of acid stress on cell growth of these mutant strains, the growth profiles were compared at pH 7.0, pH 5.0, and pH 4.5. At pH 7.0, the three mutant strains exhibited similar levels of growth and lactate concentration to that of the parent strain (Fig. 1b, S2a), whereas at pH 5.0 and pH 4.5, the strains exhibited a significant growth defect, and the parent strain showed almost no growth. These results showed that the mutant strains enabled growth beyond that of the parent strain under acid stress conditions, with *L. lactis* WH101 showing the highest final biomass and lactate concentration among the four strains (Fig. 1c, d, S2b, c).

To further evaluate the acid stress tolerance of the mutant strains, their survival rates were measured at lethal pH 4.0. Three mutant strains showed different survival rates after acid shock for various times and exhibited enhanced acid resistance, with differences in survival times increasing significantly along with extended acid shock time. After 3 h of acid shock, *L. lactis* WH101, *L. lactis* WH102, and *L. lactis*

WH103 showed 22.4-, 2.2-, and 1.9-fold higher survival rates, respectively, compared to the parent strain. Moreover, after 5 h of acid shock, the survival rates of mutant strains were markedly higher than that of the parent strain (16,000-, 351.4-, and 264.1-fold, respectively) (Fig. 1e). We further investigated the stress tolerance of the mutant strains to solvent (15% ethanol), osmotic (15% NaCl), and oxygen (1 mM H_2O_2) stresses (Fig. S3). During these three stresses, the mutant strain *L. lactis* WH101 showed 5.2-, 2.0-, and 1.9-fold higher survival rates than that of the parent strain, respectively. These results indicated that *L. lactis* WH101 displayed the best tolerance to acid and other stresses as compared with the other two mutant strains. Therefore, *L. lactis* WH101 was used for subsequent experiments.

Physiological characteristics of the selected mutant strain *L. lactis* WH101

We also investigated the physiological characteristics of the parent and *L. lactis* WH101 strains during acid stress. Time course measurements of intracellular ATP concentration for both strains (Fig. 2a) showed that ATP concentration in both strains peaked at 2 h and was nearly 2.0-fold higher than that at initiation of the experiment, followed by a gradual decline. Notably, after 6 h of acid shock, the mutant strain displayed higher ATP level that was 25% higher than that in the parent strain. These results demonstrated that the mutant strain displayed an enhanced capability to maintain elevated ATP concentrations during acid stress. Ammonia can neutralize intracellular protons during acid stress. Therefore, we investigated changes in intracellular ammonia concentration during acid stress (Fig. 2b). After 4 h of acid shock, *L. lactis* WH101 showed a 62.2% increase compared to that of the parent strain in NH_4^+ level. pH_i homeostasis plays a critical role in the cellular response to acid stress in *L. lactis*; therefore, we measured pH_i under different acid stress conditions (Fig. 2c). Incubation at pH 7.0 and pH 6.0 for 4 h resulted in no clear differences between the parent and mutant strains; however, further decreasing the pH of acid stress to pH 5.0 caused the reduction of pH_i of both strains, although the mutant strain showed a higher pH_i value than the parent strain. These results suggested that *L. lactis* WH101 maintained a higher NH_4^+ concentration and a relatively stable pH_i during acid stress.

Most acid stress-response processes need to consume additional energy to guarantee the growth of microbial cells. Protons expelled from cells need to consume ATP, whereas some amino acid metabolic pathways produce ATP and ammonia, which can subsequently neutralize intracellular protons to maintain relative pH_i stability [20]. Additionally, pH_i homeostasis plays a critical role in maintaining normal physiological stability in microorganisms during acid stress [17]. pH_i is affected by changes in external pH, which can influence the relative balance of intracellular metabolism and especially the activity of certain enzymes [12]. In this study, we observed that *L. lactis* WH101 harbored higher ATP and NH_4^+ concentrations than those in the parent strain, while also maintaining a higher pH_i level compared with the parent strain. We also found that intracellular ATP concentrations increased within the first 2 h of stress before gradually decreasing. This might be due to the sensing process of cells in the early stage of stress, which allows the production of additional ATP in response. Therefore, the ability of the mutant strain to maintain higher ATP, NH_4^+, and pH_i levels improved the adaptability of the cells to an acidic environment.

Comparative transcriptome analysis of the parent and mutant strains during acid stress

To investigate possible acid stress-response mechanisms in *L. lactis*, we performed transcriptome sequencing to

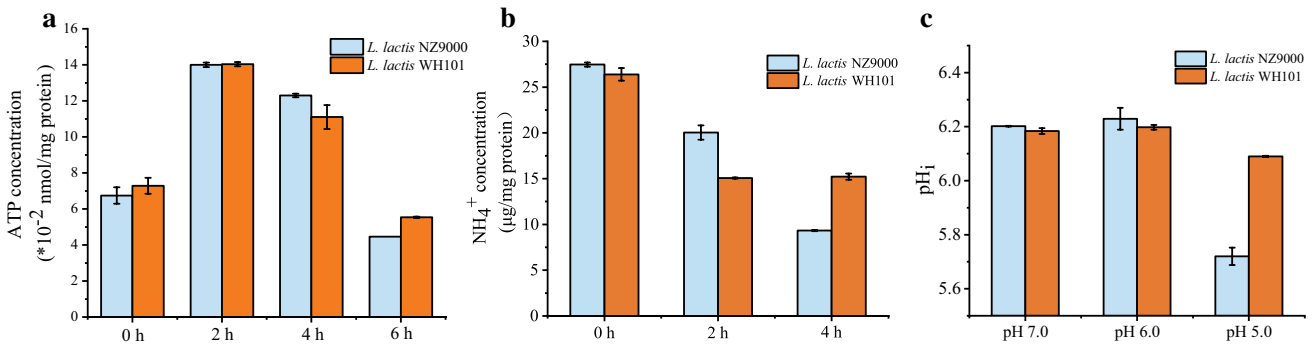

Fig. 2 Physiological characteristics of the parent strain and *L. lactis* WH101. Changes in intracellular ATP concentration (**a**), NH_4^+ concentration (**b**), and pH_i (**c**) during acid stress. Both strains were exposed to acid stress at pH 5.0 for various times (**a**, **b**). For pH_i determination, strains were exposed to acid stress at pH 6.0 and pH 5.0 for 4 h. Error bars represent the mean ± standard deviation of three replicates

compare different gene expression patterns between the parent and mutant strains at 0 and 4 h. Differentially expressed genes were analyzed in four comparison groups (Fig. S4) as follows: (1) *L. lactis* NZ9000 and *L. lactis* WH101 at 4 h; (2) *L. lactis* NZ9000 and *L. lactis* WH101 at 0 h; (3) *L. lactis* NZ9000 at 0 and 4 h; and (4) *L. lactis* WH101 at 0 and 4 h (Supplementary Excel Files_1). Additionally, a subset of 61 genes appeared across the four comparison groups (Fig. S5).

Based on gene ontology analysis, genes differentially expressed between the comparison groups were mainly involved in metabolic and cellular processes, as well as catalytic activity and binding (Fig. S6). KEGG enrichment analysis showed that carbohydrate metabolism, amino acid metabolism, and fatty acid metabolism were the three most highly enriched pathways (Fig. S7). Sugar transporters, nitrogen metabolism, and biosynthesis of secondary metabolites represented the second-most notable enrichment pathways. These results revealed that carbohydrate metabolism, amino acid metabolism, and fatty acid metabolism were strongly affected by acid stress. Based on the transcriptome results, nine genes were selected for verification by qRT-PCR analysis, which revealed positive correlations with the transcriptome data between the four comparison groups (Fig. S8).

Analysis of acid stress response by the parent and mutant strains

We subsequently analyzed the expression profiles of genes involved in carbohydrate metabolism, sugar transport, amino acid metabolism and transport, and fatty acid metabolism (Fig. 3). For the carbohydrate metabolism pathways, *bglX* (beta-glucosidase), *malQ* (4-alpha-glucanotransferase), and *kdgA* (keto-hydroxyglutarate-aldolase) genes were upregulated in the parent strain during acid stress, while *malQ*, *gltA* (citrate/2-methylcitrate synthase) and *kdgA* genes were downregulated in the mutant strain. Moreover, we observed that *gpmA* (2,3-bisphosphoglycerate-dependent

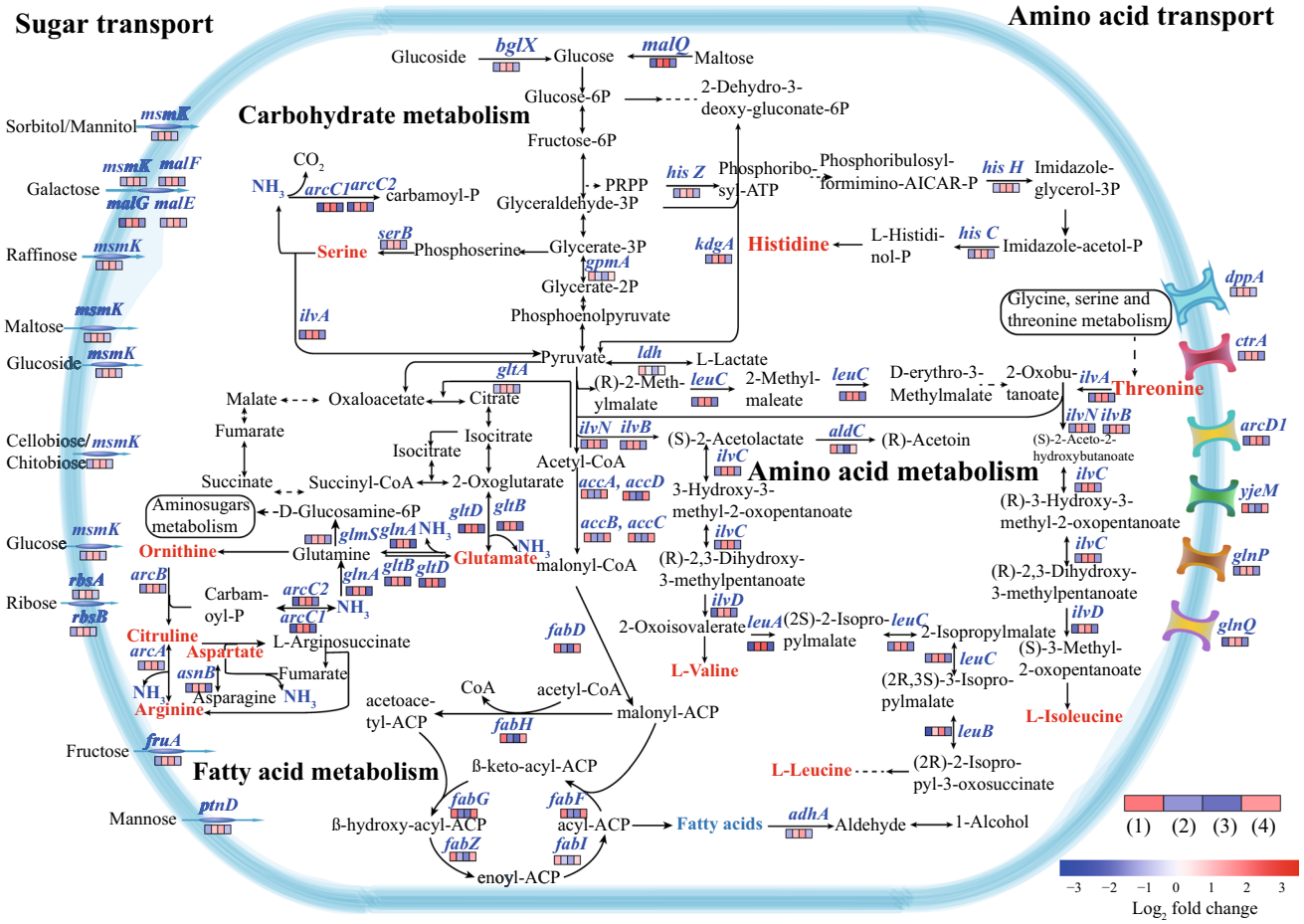

Fig. 3 Schematic representation of the transcription levels of differentially expressed genes involved in carbon metabolism, amino acid metabolism, fatty acid metabolism, and transporters. Each gene is accompanied by its expression ratio (log_2-fold change). Comparisons included the following: (1) *L. lactis* WH101 compared with *L. lactis* NZ9000 at 4 h; (2) *L. lactis* WH101 compared with *L. lactis* NZ9000 at 0 h; (3) *L. lactis* NZ9000 at 4 h compared with that at 0 h; and (4) *L. lactis* WH101 at 4 h compared with that at 0 h

phosphoglycerate mutase) and *ldh* (malate/lactate dehydrogenases) genes exhibited repressed expression in the parent strain. Several genes related to sugar transport were considerably induced. During acid stress, genes involved in multi-sugar transport (*msmK*, *malEFG*) and ribose (*rbsA* and *rbsB*), fructose (*fruA*), and mannose (*ptnD*) transport were also upregulated in the parent strain. Meanwhile, the *malG* gene was also upregulated in the mutant strain under unstressed condition.

Carbohydrate metabolism is vital for acid tolerance in *L. lactis*, because it represents the primary pathway allowing cells to generate energy [4]. Cells consume increased amounts of ATP in response to acid stress, and the energy from sugar metabolism is mainly applied to cope with the harsh acid environment [25]. Therefore, the activation of carbon metabolic pathways enhances cell growth and acid stress-tolerance capacity. In this study, the expression of genes involved in carbon metabolic pathways and those encoding sugar transporters were significantly influenced in the parent strain, which attributed to the promotion of ATP synthesis.

We observed that genes involved in the metabolism of serine, histidine, isoleucine, leucine, valine, arginine, aspartate, and glutamate were particularly upregulated in the parent strain during acid stress (Fig. 3). These included genes involved in histidine biosynthesis (*hisCHZ*); serine biosynthesis and metabolism (*serB*, *ilvA* and *arcC1C2*); leucine, isoleucine, and valine biosynthesis (*ilvBCDN* and *leuABC*); glutamate (*gltBD*, *glmS* and *glnA*), arginine (*arcABC1C2*), and aspartate (*asnB*) metabolism. Meanwhile, several genes involved in amino acid transport (*dppA*, *ctrA*, *arcD1* and *glnPQ*) were also upregulated in parent strain during acid stress.

Amino acids mainly participate in pH_i homeostasis, ATP generation, and protein synthesis, which are intimately involved in regulating acid tolerance [5]. The arginine deiminase (ADI) pathway generates ammonia and ATP through conversion of arginine to ornithine via citrulline and can maintain relative pH_i homeostasis by neutralizing protons and promoting extracellular transfer of H^+ via proton pumps [18]. Subsequently, glutamate contributes to acid stress tolerance through the glutamate decarboxylase (GAD) system, where glutamate and intracellular protons are decarboxylated to generate gamma-aminobutyric acid and CO_2 and maintain pH_i through proton consumption [3]. Moreover, we found that aspartate was related to acid stress response in bacteria, which also agreed with a previous report [21].

A total of nine genes implicated in fatty acid metabolism (*accABCD* and *fabDFGHZ*) were prominently upregulated in the mutant strain during acid stress, whereas a gene associated with fatty acid degradation (*adhA*) was downregulated. Overall, these results suggested that *L. lactis* mainly regulated carbohydrate metabolism, amino acid transport and metabolism, and sugar transport in response to acid stress.

Transcriptional regulation is a common mechanism that occurs in response to acid stress [14], and a range of transcriptional regulators have been identified as contributing to acid tolerance. In *L. lactis*, *ythA* (a PspC-family transcriptional regulator) increases acid stress tolerance and nisin yield [23]. Moreover, *ccpA* and *codY* optimize acetate metabolism in response to environmental pH by regulating transcription of *ackA* and *pta* in *Streptococcus mutans* [11]. In the present study, we identified transcriptional regulators [*cmhR* (LysR family transcriptional regulator), *itrBE* (transcription elongation factor GreAB), and *traD* (conjugal transfer protein)] potentially contributing to acid stress response (Supplementary Excel Files_1).

System-level investigation of *L. lactis* acid tolerance suggested several response regulation mechanisms. Therefore, we proposed an acid stress-response model for *L. lactis* (Fig. S9) that involved the following pathways and activities: (1) transport of sugar, amino acids, ions, and membrane proteins; (2) biosynthesis of amino acids and fatty acids; (3) carbohydrate and amino acid metabolism; (4) transcriptional regulation; and (5) activation of stress-response genes, such as those encoding molecular chaperones. Our findings provide valuable insights into the mechanisms associated with acid stress response in *L. lactis* and promote further development of strains exhibiting enhanced acid stress tolerance.

Effects of overexpression of anti-acid components on acid tolerance in *L. lactis*

Based on transcriptome analysis, carbon and amino acid metabolism are important factors that influence acid stress tolerance. Therefore, two significantly differentially expressed genes *arcB* (involved in amino acid metabolism) and *malQ* (involved in carbon metabolism) were selected for overexpression in *L. lactis* NZ9000 to improve its acid stress tolerance. To further investigate the effects of acid stress on these recombinant strains, acid stress-tolerance assays were conducted. The results showed that the recombinant strains showed increased acid stress tolerance as compared with the control strain (Fig. 4). After acid shock for 3 h at pH 4.0, *L. lactis* (ArcB) and *L. lactis* (MalQ) exhibited 12.6- and 12.9-fold higher survival rates than that of the control strain, respectively, indicating that overexpression of these genes enhanced the acid stress tolerance of *L. lactis*.

Conclusions

In this study, genome mutagenesis was combined with the application of high-throughput technologies to identify acid stress-tolerant strains. The mechanism by which the

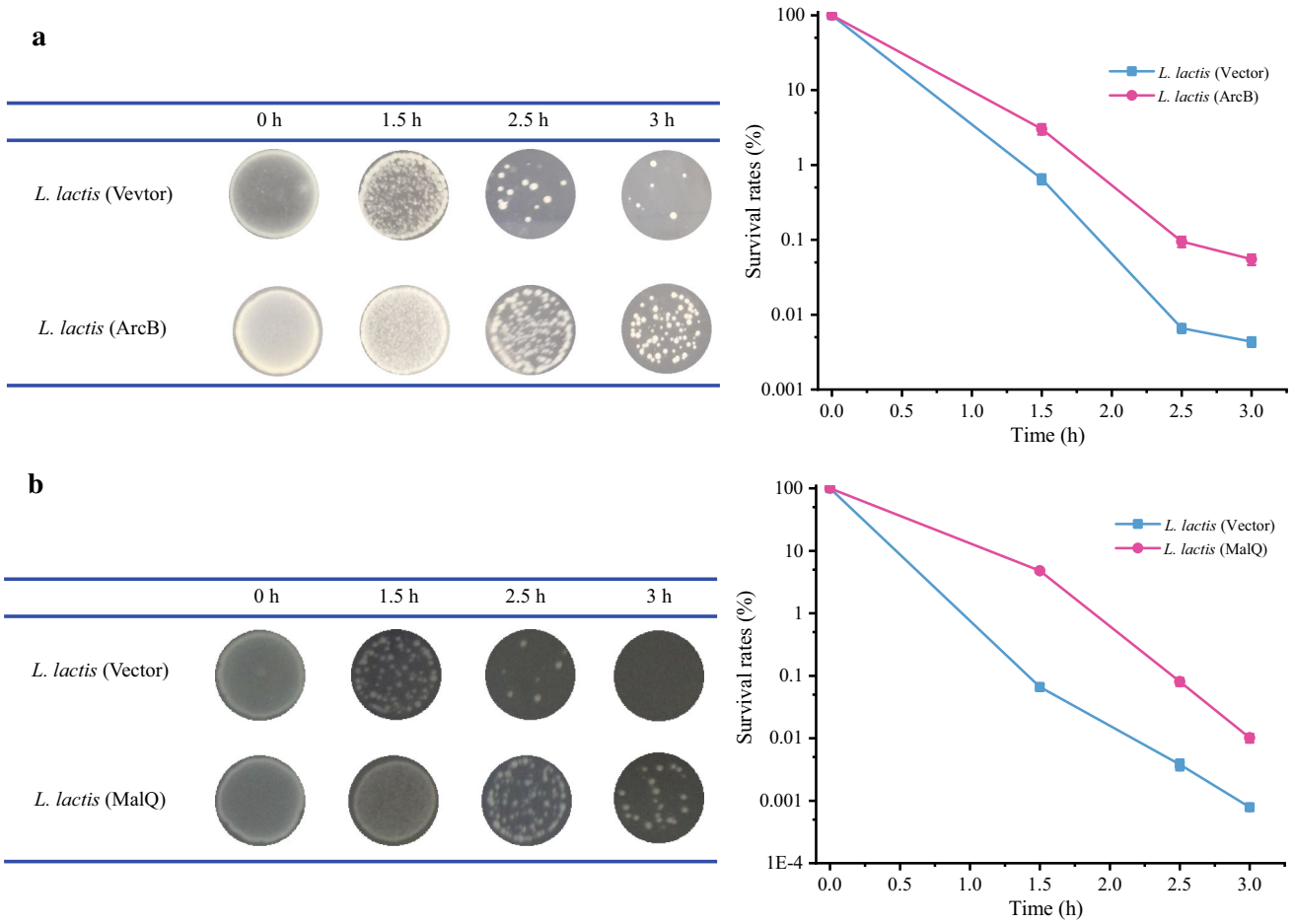

Fig. 4 Influence of overexpressed acid stress-response components on *L. lactis* viability during acid stress. The genes *arcB* (**a**) and *malQ* (**b**) were overexpressed, followed by determination of cell survival rates after exposure to acid stress at pH 4.0 for various times. *L. lactis* (Vector) was the control strain (containing the empty pNZ8148 plasmid). Error bars represent the mean ± standard deviation of three replicates

L. lactis acid-tolerance response is regulated was systematically investigated, and an acid stress-response model for *L. lactis* was proposed. Then, two identified anti-acid components were overexpressed, and acid tolerance was analyzed. Our findings provide valuable insights into the mechanisms associated with the acid stress response in *L. lactis* and promote the further development of strains exhibiting enhanced acid stress tolerance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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