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ORIGINAL PAPER

Transcriptome Analysis of *Komagataeibacter europaeus* CGMCC 20445 Responses to Different Acidity Levels During Acetic Acid Fermentation

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Abstract

In the industrial production of high-acidity vinegar, the initial ethanol and acetic acid concentrations are limiting factors that will affect acetic acid fermentation. In this study, *Komagataeibacter europaeus* CGMCC 20445 was used for acetic acid shake flask fermentation at an initial ethanol concentration of 4.3% (v/v). We conducted transcriptome analysis of *K. europaeus* CGMCC 20445 samples under different acidity conditions to elucidate the changes in differentially expressed genes throughout the fermentation process. We also analyzed the expression of genes associated with acid-resistance mechanisms. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis showed that the differentially expressed genes were enriched in ribosomes, citrate cycle, butanoate metabolism, oxidative phosphorylation, pentose phosphate, and the fatty acid biosynthetic pathways. In addition, this study found that *K. europaeus* CGMCC 20445 regulates the gene expression levels of cell envelope proteins and stress-responsive proteins to adapt to the gradual increase in acidity during acetic acid fermentation. This study improved the understanding of the acid resistance mechanism of *K. europaeus* and provided relevant reference information for the further genetic engineering of this bacterium.

K e y w o r d s: acetic acid bacteria, *Komagataeibacter europaeus*, acid resistance, transcriptomics

Introduction

Acetic acid bacteria are a large family of aerobic gram-negative bacteria that use oxygen as a terminal electron acceptor. During oxidation, these bacteria convert ethanol and sugars into aldehydes, ketones, and organic acids (Wang et al. 2015). Currently, 19 genera and 92 species of acetic acid bacteria have been discovered (Qiu et al. 2021). The conversion of ethanol into acetic acid by acetic acid bacteria has broad applications. For example, wine vinegar is used as a food flavoring agent and preservative (Tesfaye et al. 2002). The daily consumption of suitable volumes of vinegar provides resistance to some infectious diseases and has positive effects on diabetes, cancer, heart disease, hypertension, and hyperlipidemia (Ali et al. 2016; Samad et al. 2016). In order to save storage space and transportation costs, there is a high commercial demand for the production of high-acidity vinegar (acetic acid concentration ≥ 9% (v/v)) (Qi et al. 2014).

Aerobic submerged fermentation is the primary liquid-state acetic acid fermentation method used in industrial vinegar production and has advantages such as high yield and high efficiency (Gullo et al. 2014). The limiting factors of aerobic submerged fermentation include the initial ethanol and acetic acid concentrations. During the initial phase of fermentation, a high ethanol concentration will affect the growth of acetic acid bacteria (Chen et al. 2016). In addition, when the acetic acid concentration is 0.5% (v/v), the intracellular dissociation of acetic acid will decrease the pH and result in metabolic disturbances, which is toxic to acetic acid bacteria (Trček et al. 2015; Xia et al. 2016). *Acetobacter pasteurianus,* which is commonly used in industrial production, is a fermenting strain used for low-acidity vinegar (acetic acid concentration < 6% (v/v)).

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Even *A. pasteurianus* isolated from bioreactors used for high-acidity vinegar fermentation does not exhibit good tolerance towards high acetic acid concentrations (Gullo et al. 2014). Sievers and coworkers were the first to isolate *K. europaeus* from high-acidity fermentation broth (Yamada et al. 2012). *K. europaeus* can grow in highly acidic environments with acetic acid concentrations of 10–14% (v/v) and is frequently used in the industrial fermentation of high-acidity vinegar (Gullo et al. 2014). Therefore, studying the acid-resistance mechanisms of *K. europaeus* during acetic acid fermentation is important.

The transcriptome is the sum of the transcripts expressed by an organism, tissue, or cell within a specific time point or environmental condition. Transcriptome research is vital to understanding gene function and its metabolic mechanisms (Filiatrault 2011). Prokaryotes are exposed to various environments and regulate the expression of corresponding genes to respond to environmental changes and stress. Hence, transcriptome analysis can accurately and effectively reveal the mechanisms of biological responses to environmental changes in prokaryotes (Nguyen et al. 2007; Benjak et al. 2016; Clauss-Lendzian et al. 2018). With the development of sequencing technology, prokaryotic transcriptome sequencing and analysis has matured. Sakurai et al. (2011) studied the transcriptomes of *Acetobacter aceti* NBRC 14818 using ethanol, acetate, and glucose as the carbon source and found differences in transcript levels of genes in the central carbon metabolic pathway and respiratory chain under different carbon source conditions. Yang et al. (2019) used *A. pasteurianus* CGMCC 1.41, which is commonly used for vinegar fermentation, to test fermentation under different initial ethanol concentrations, using transcriptome sequencing to examine the acid-resistance mechanisms. Ryngajłło et al. (2019) used the RNA-seq technology to study the effect of adding ethanol to the culture medium on the gene expression profile of *Komagataeibacter xylinus* E25.

We used *K. europaeus* CGMCC 20445 for acetic acid shake flask fermentation. Sampling was carried out under different acetic acid concentrations for prokaryotic transcriptome sequencing and analysis. This was used to examine its acid-resistance mechanisms, which could be used to inform genetic engineering to increase its acetic acid yield.

Experimental

Materials and Methods

Culture medium. The GY_1 culture medium (1% glucose, 1% yeast powder, and 3% anhydrous ethanol) was used for acetic acid bacteria activation and the preparation of the seed culture. The GY_2 culture medium (1% glucose, 1% yeast powder, 0.06% KH_2PO_4, and 0.6% $MgSO_4 \cdot H_2O$) was used to inoculate the seed culture for acetic acid fermentation.

Strain activation and fermentation. An inoculating loop was used to inoculate *K. europaeus* CGMCC 20445 (from the China General Microbiological Culture Collection Center) in a 250 ml conical flask containing 50 ml GY_1. The flask was cultured at 30°C with shaking at 200 r/min for 72 h to prepare the seed culture. Ten milliliters of seed culture were added to 500 ml conical flasks containing 100 ml GY_2, after which 4.3% (v/v) ethanol and 0.9% (v/v) acetic acid were added. The shaking incubator temperature and speed were set at 30°C and 200 r/min, respectively, to initiate acetic acid fermentation. Phenolphthalein was used as a pH indicator and 0.1 mol/l sodium hydroxide titration was used to measure the acidity in the acetic acid fermentation broth. An ultraviolet-visible spectrophotometer (Shimadzu Instruments Manufacturing Co., Ltd., Suzhou, China) was used to measure cell growth at a wavelength of 600 nm.

Sampling. At 16 h, 40 h, 64 h, and 88 h, 4 ml of bacterial culture was collected and added to the centrifuge tube. The acidity of the samples was measured, and samples were centrifuged at 4°C and 5,000 r/min for 10 min. The supernatant was discarded, and samples were immediately stored at –80°C in a freezer. The samples used for the transcriptome sequencing were named z16h, z40h, z64h, and z88h, and each sample had three biological replicates.

RNA extraction, quantitation, and identification. Total RNA was extracted using TRIzol® Reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Agarose gel electrophoresis (1%) was used to examine the RNA degradation and contamination in the sample. A NanoPhotometer® was used to determine the RNA purity. The Qubit® RNA Assay Kit in the Qubit® 2.0 Fluorometer was used to measure the RNA concentration. The RNA Nano 6000 Assay Kit in the Agilent Bioanalyzer 2100 system was used to assess RNA integrity.

RNA sequencing and data analysis. Once the quality of the samples was confirmed, the Ribo-zero kit was used to remove rRNA and enrich mRNA. Following that, fragmentation buffer was added to break up the mRNA into short fragments. The mRNA was used as a template, and random hexamers were used to synthesize the first cDNA strand. Following that, buffer, dNTPs (dUTP replaced dTTP in dNTPs), DNA polymerase I, and RNase H were used to synthesize the second cDNA strand before the AMPure XP system was used to purify double-stranded cDNA. Following that, the USER Enzyme was used to degrade the uracil-containing second cDNA strands. The terminal repair was first carried out on purified double-stranded cDNA, and an A-tail

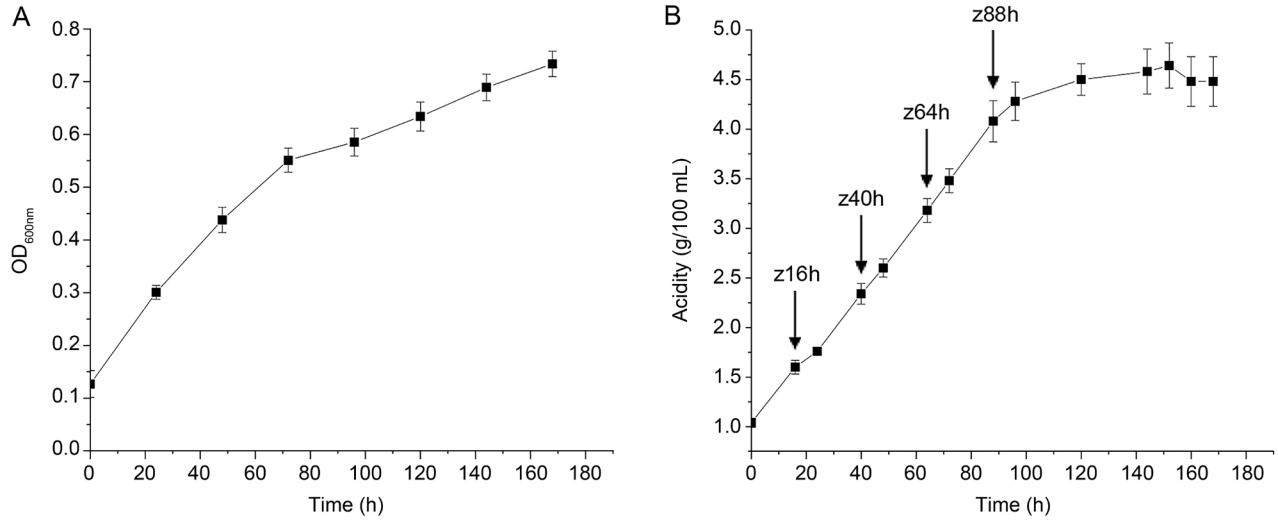

Fig. 1. Acetic acid fermentation of *Komagataeibacter europaeus* CGMCC 20445 at an initial ethanol concentration of 4.3% (v/v).
A) The cell growth of *K. europaeus* CGMCC 20445. B) Changes in the acidity of the fermentation medium. The arrow indicates the sample corresponding to the transcriptome sequencing. Data obtained from three biological replicates were shown as mean ± standard deviation.

and adapter sequences were added. The AMPure XP system was then used to screen for cDNA fragments with 150–200 bp lengths. Finally, PCR amplification and the AMPure XP system were used to purify PCR products to obtain the final libraries. The Agilent Bioanalyzer 2100 system was used to measure library quality. TruSeq PE Cluster Kit v3-cBot-HS (Illumina) was used to cluster index-coded samples in the cBot Cluster Generation System. After clusters were synthesized, the Illumina HiSeq platform was used for library sequencing and generation of paired-end reads. The clean reads were aligned to the reference genome (accession no. CP021467.1) using Rockhopper 2 (Tjaden 2015). The gene expression data generated during this study were submitted to the Gene Expression Omnibus (GEO) database of NCBI; GEO accession number GSE168432 was assigned to these data. FPKM (expected number of Fragments Per Kilobase of transcript sequence per Million base pairs sequenced) is currently the most commonly used method for estimating gene expression levels (Trapnell et al. 2010). An FPKM > 1 means that the gene is expressed. DESeq R package (1.18.0) software was used for the analysis of differential expression of genes (Anders and Huber 2010) and the criterion for selecting differentially expressed genes was padj < 0.05. The GOseq R package was used for Gene Ontology (GO) enrichment analysis and the correction of gene length errors in the differentially expressed genes (Young et al. 2010). A corrected *p*-value < 0.05 indicates that the differentially expressed gene is significantly enriched for that GO term. We used KOBAS (2.0) software to test the statistical enrichment of differential expression genes in KEGG pathways. A *p*-value ≤ 0.05 indicates that the differentially expressed gene is significantly enriched for that pathway.

Results and Discussion

Acetic acid fermentation. When *K. europaeus* CGMCC 20445 was subjected to acetic acid fermentation, the initial acidity of the medium was 1.04 ± 0.03 g/100 ml, and the maximum acidity of the medium was 4.64 ± 0.23 g/100 ml (Fig. 1). The samples z16h, z40h, z64h, and z88h used for transcriptome sequencing were sampled in medium with the acidity of 1.60 ± 0.07 g/100 ml, 2.34 ± 0.10 g/100 ml, 3.18 ± 0.12 g/100 ml, and 4.08 ± 0.21 g/100 ml, respectively.

Analysis of the expression levels of differentially expressed genes and GO enrichment analysis results. The Pearson correlation coefficient (R) is used to express the correlation of gene expression levels between samples. In this study, the R^2 between samples was close to 1 (Fig. S1), indicating that the experiment was reliable, and the sample selection was reasonable. As acidity increased during fermentation, the total numbers of differentially expressed genes at 40 h, 64 h, and 88 h in comparison to 16 h were 908, 979, and 1,222, respectively; there were 502, 500, and 602 upregulated genes, respectively, and 406, 479, and 620 downregulated genes, respectively. From 40 h to 88 h, the ratio of differentially expressed genes in the genome increased from 26% to 35%, and the ratios of upregulated genes and downregulated genes both increased (Table I). The GO enrichment analysis showed that only downregulated genes were significantly enriched (Table SI), and no significant enrichment occurred in the upregulated genes. These down-regulated genes encode for many cellular components of ribosomes, the proteins involved in the molecular functions of the ribosome, proteins involved in transmembrane transport, and oxidoreductases, as well as those participating in the biological processes

Table I
Statistics of differentially expressed genes of the samples treated.

Time	Number of up-regulated genes	Number of down-regulated genes differentially expressed genes	
40 h	502[a] (14%)	406 (12%)	908 (26%)
64 h	500 (14%)	479 (14%)	979 (28%)
88 h	602 (17%)	620 (18%)	1,222 (35%)

[a] – percentages shown in the brackets indicate the proportion that accounts for total genes within the genome

of protein and organic nitrogen compound synthesis and metabolism.

Analysis of the expression status of genes associated with acid resistance. We carried out a KEGG pathway enrichment analysis of *K. europaeus* CGMCC 20445 to examine the effects of the continuous increase in acetic acid concentration during fermentation on metabolic pathways in acetic acid bacteria. We found that compared with 16 h, ribosome genes were significantly downregulated at 40 h. When the transcriptome at 64 h was compared with that at 16 h, genes in the citrate cycle, oxidative phosphorylation, and butanoate metabolism were significantly upregulated, while ribosome and fatty acid biosynthesis genes were significantly downregulated. When the transcriptome at 88 h was compared with that at 16 h of fermentation, genes in the citrate cycle, butanoate metabolism, pentose phosphate pathway, and oxidative phosphorylation were significantly upregulated, while ribosome genes were significantly downregulated (Table SII). The following section will explore the expression levels of genes in these significantly enriched metabolic pathways, ethanol oxidation, cell envelope proteins, and stress-responsive proteins, and examine the corresponding acid-resistance mechanisms.

Ribosomes. In this study, as fermentation duration increased, the number of downregulated genes related to ribosomes continuously increased. Table SIII.1 shows the expression levels of differentially expressed genes related to ribosomes. Increases in acetic acid acidity have adverse effects on ribosome integrity and protein biosynthesis (Xia et al. 2016). Andrés-Barrao et al. (2012) studied the proteome of *A. pasteurianus* during acetic acid fermentation and found that most ribosomal proteins were downregulated in the late fermentation stage as ethanol concentration decreased and acetic acid concentration increased.

Ethanol oxidation. Pyrroloquinoline quinone-dependent alcohol dehydrogenase (PQQ-ADH) and pyrroloquinoline quinone-dependent aldehyde dehydrogenase (PQQ-ALDH) constitute the ethanol respiratory chain, which converts ethanol to acetic acid through incomplete oxidation (Matsushita et al. 2004). PQQ-ADH activity is associated with acid resistance in acetic acid bacteria (Trcek et al. 2006). Acetic acid bacteria can use the energy released from the incomplete oxidation of ethanol in this process (Gomes et al. 2018). *A. pasteurianus* PQQ-ADH is composed of three subunits: subunit I, encoded by *adhA*, includes pyrroloquinoline quinone and one heme c; subunit II, encoded by *adhB*, contains three heme c and is related to membrane-bound ubiquinone reduction; and subunit III, encoded by *adhS*, is not essential for ethanol oxidation (Kondo et al. 1995; Toyama et al. 2004; Wu et al. 2017). Bacteria genetically engineered to overexpress *adhA* and *adhB* in PQQ-ADH have a 17% higher acetic acid yield than the parental strain under identical conditions (Wu et al. 2017). Quinoprotein ADH-deficient mutants completely lose the ability to synthesize and tolerate acetic acid (Chinnawirotpisan et al. 2003). Strains with *adhA* gene disruption show decreased acetic acid tolerance and decreased ADH activity (Chinnawirotpisan et al. 2003). Table SIII.2 shows the expression levels of genes related to ethanol oxidation. The expression levels of *adhA* (S101446_02993) and *adhB* (S101446_02992) genes were significantly downregulated at 40 h and returned to levels close to that observed at 10 h by 64 h and 88 h. The gene expression levels of *adhA* and *adhB* were high throughout the fermentation process, which showed that PQQ-ADH was crucial for acetic acid fermentation. There was no significant change in the gene expression level of PQQ-ALDH in this study. NAD-dependent alcohol dehydrogenase and NAD-dependent acetaldehyde dehydrogenase in the cytoplasm can also incompletely oxidize ethanol to acetic acid. The expression of NAD-dependent acetaldehyde dehydrogenase in the cytoplasm is inhibited in a medium containing ethanol (Andrés-Barrao et al. 2012). In this study, the NAD-dependent acetaldehyde dehydrogenase gene *aldh2* (S101446_00292) was continuously downregulated during fermentation (Fig. 2), and the expression level of the NAD-dependent alcohol dehydrogenase gene did not change significantly. This indicates that the increase in acidity inhibits the expression of NAD-dependent acetaldehyde dehydrogenase of this strain.

Acetic acid assimilation and butanoate metabolism. Acetic acid can be directly converted by *aarC* or *acs* to acetyl CoA or be converted by *ackA* to acetyl-P before being converted by *pta* to acetyl CoA and entering the citrate cycle (Wang et al. 2015). In this study, the expression levels of *acs* (S101446_00552) and *ackA* (S101446_02246, S101446_02247) continuously increased, and the expression level of *pta* (S101446_02900) was upregulated at 88 h (Fig. 2). A study on the mutant *A. pasteurianus* found that *aarC* is an acetic acid tolerance gene that is associated with an enzyme encoded in the citrate cycle. The gene *aarC* encodes for succinyl CoA: acetate CoA-transferase that

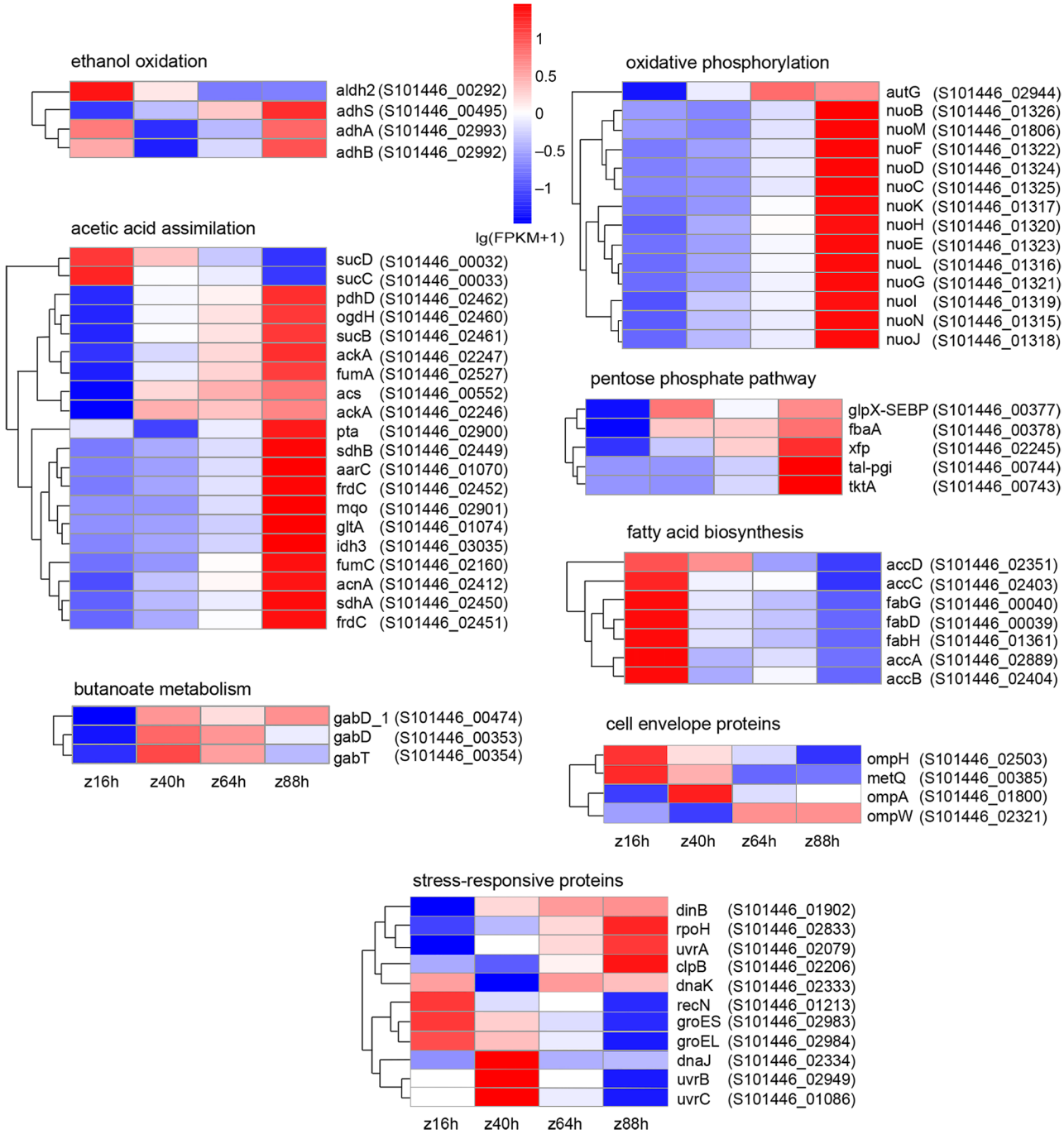

Fig. 2. Heatmap of differentially expressed genes. The values of lg (FPKM+1) of genes in different samples are normalized, and the genes are clustered. The upregulated and downregulated genes were represented by red and blue grids, respectively. Table S3 shows the names of each gene, fkpm, padj, and the annotation information.

converts succinyl CoA to succinate and converts intracellular acetic acid to acetyl CoA to enter the citrate cycle (Fukaya et al. 1993; Mullins et al. 2008). In this study, the expression level of *aarC* (S101446_01070) continuously increased from 64 h onwards (Fig. 2). The expression levels of most enzyme genes associated with the citrate cycle increased as acidity increased during fermentation, and only *sucCD*, which is related to succinyl-CoA synthase, was downregulated, whereas *sucC* (S101446_00033) was continuously downregulated and *sucD* (S101446_00032) was downregulated at 88 h (Fig. 2). Aconitase is an enzyme in the citrate cycle that converts citrate to isocitrate. Nakano et al. (2004) overexpressed aconitase in *A. aceti* and found that this could increase aconitase activity and acetic acid resistance in that strain. They found that acetic acid yield and acid resistance in the aconitase-overexpressing strains were better than in the parental strain and could reach the logarithmic growth phase and stationary phase earlier during fermentation, and thus increased the acetic acid

yield by 25% compared with the parental strain (Nakano et al. 2004). The gene *acnA* (S101446_02412) encodes for aconitase, and its expression was continuously upregulated as acidity increased during fermentation (Fig. 2). Table SIII.3 shows the expression levels of genes associated with acetic acid assimilation. In butanoate metabolism, 4-aminobutanoate is converted to succinate semialdehyde, which is then converted into succinate. The expression levels of genes in this metabolic pathway were upregulated (Fig. 2). Increases in succinate facilitate the progression of the citrate cycle. Table SIII.4 shows the expression levels of genes in this pathway.

Oxidative phosphorylation. *A. pasteurianus* was separately cultured in an ethanol-containing and ethanol-free medium (Yang et al. 2019). Compared with the culture without ethanol, ethanol dehydrogenase and acetaldehyde dehydrogenase were continuously upregulated in the ethanol-containing culture during fermentation, while NADH dehydrogenase complex (complex I) and succinate dehydrogenase complex (complex II) were continuously downregulated in the ethanol-containing culture during fermentation (Yang et al. 2019). At the middle stage and late stage of fermentation, ethanol dehydrogenase and acetaldehyde dehydrogenase were upregulated, while the downregulation of NADH dehydrogenase complex (complex I) and succinate dehydrogenase complex (complex II) showed a decreasing trend (Yang et al. 2019). This showed that oxidative phosphorylation and the ethanol respiratory chain have a complementary relationship in the energy supply in *A. pasteurianus*. In this study, at 64 h and 88 h of fermentation, oxidative phosphorylation was significantly enriched and NADH dehydrogenase complex (complex I) and succinate dehydrogenase complex (complex II) were significantly upregulated compared with the early stage of fermentation (Fig. 2). Table SIII. 5 shows the expression levels of genes associated with oxidative phosphorylation. Additionally, there was almost no change in the expression level of ATP synthase. It indicates that oxidative phosphorylation may not complement the ethanol respiratory chain for energy supply at the late stage of fermentation, and the ethanol respiratory chain may still be the primary energy source.

Pentose phosphate pathway and fatty acid biosynthesis. The pentose phosphate pathway produces pentose phosphate for efficient nucleic acid synthesis and provides NADPH, essential for fatty acid synthesis and cell survival under stressful conditions. In this study, as acidity increased during fermentation, genes associated with the pentose phosphate pathway were upregulated (Fig. 2). Table SIII.6 shows the expression levels of genes associated with pentose phosphate pathway. Glucose was continuously consumed during fermentation, and pentose phosphate pathway genes were continuously upregulated during fermentation in *A. pasteurianus* (Yang et al. 2019). The structure and chemical composition of the cell membrane affects acid resistance in acetic acid bacteria. This is associated with fatty acid synthesis (Higashide et al. 1996; Goto et al. 2000). In this experiment, genes associated with fatty acid synthesis showed overall downregulation as acidity increased (Fig. 2). Table SIII.7 shows the expression levels of genes associated with fatty acid biosynthesis. Both the gene *fabD* (S101446_00039) and the gene *fabG* (S101446_00040) were also downregulated (Fig. 2), which is consistent with the results of Yang et al. (2019). The gene *fabD* will affect the initial reaction of fatty acid biosynthesis in bacteria and provide malonyl groups for polyketone biosynthesis. The gene *fabG* participates in fatty acid chain elongation. Therefore, the downregulation of *fabD* and *fabG* will weaken the flux through the fatty acid pathway, thereby reducing total lipid content. Changes in lipid content in the cell membrane will affect the ability of the cell membrane to buffer acetic acid molecules (Xia et al. 2016).

Cell envelope proteins. OmpA is a central component of the outer membrane and plays a role as a pore protein. OmpA has non-specific low permeability towards small solutes and maintains a stable outer membrane structure (Confer and Ayalew 2013; Xia et al. 2016). OmpH is also an important outer membrane protein, and large amounts of OmpH are present on bacteria surfaces. OmpH acts as a molecular sieve, enabling small molecule hydrophilic solutes to diffuse through the outer membrane (Ganguly et al. 2015; Xia et al. 2016). The lipoprotein MetQ has important roles in maintaining outer membrane integrity and promoting cell division (Zhai et al. 2014; Xia et al. 2016). Xia et al. (2016) exposed *A. pasteurianus* to different acetic acid concentrations as stressors. When samples with an acetic acid concentration of 9.3% (w/v) were compared with samples with an acetic acid concentration of 3.6% (w/v), the membrane protein OmpA was downregulated 0.4-fold, the membrane protein OmpH was upregulated 2.23-fold, and lipoprotein MetQ was upregulated 7.74-fold. Andrés-Barrao et al. (2012) used *A. pasteurianus* LMG 1262^T for oxidative fermentation in 3.9% ethanol and 0.1% acetic acid and found that the expression of OmpA protein was downregulated in the late fermentation stage. In this study, samples from 16 h were used as controls. At 40 h, *ompA* (S101446_01800) was upregulated, while there were no significant changes to *ompH* (S101446_02503) and *metQ* (S101446_00385). From 40 h to 88 h, there was no significant change in *ompA*, while *ompH* and *metQ* were downregulated (Fig. 2 and Table SIII.8). The outer membrane protein OmpW may protect bacteria from environmental stress and can participate in the transport of small hydrophobic molecules across the bacterial outer membrane (Hong et al. 2006). The expression of the outer

membrane protein OmpW is activated in response to oxidative stress (Gil et al. 2009). In this study, the gene expression level of *ompW* (S101446_02321) was significantly upregulated at 64 h and 88 h. However, there have been no reports on OmpW protein in the acid resistance mechanism of *A. pasteurianus*. The results show that there are differences in the expression levels of outer membrane protein genes and lipoprotein genes of *A. pasteurianus* and *K. europaeus* during acetic acid fermentation, which may affect the difference in the acid resistance of *A. pasteurianus* and *K. europaeus*. Further research on this subject is needed.

Stress-responsive proteins. Heat shock proteins (HSPs) are classical stress proteins. These highly conserved proteins are selectively synthesized by cells under stress, such as stress due to high temperatures, pathogens, cytokines, or harmful physicochemical factors. HSPs regulate the correct folding and assembly of membrane proteins, prevent protein misfolding, and promote protein refolding and correct assembly (Hemmingsen et al. 1988). Okamoto-Kainuma et al. (2002) found that when *A. aceti* was cultured in environments with ethanol, acetic acid, or high temperature, the intracellular synthesis of two stress proteins, GroES and GroEL, occurred. These stress proteins are known as chaperones. Strains overexpressing GroES and GroEL can tolerate higher temperatures, ethanol concentrations, and acetic acid concentrations than the parental strain (Okamoto-Kainuma et al. 2002). The expression of chaperones such as GroES, GroEL, DnaJ, DnaK, and ClpB is regulated to varying degrees by *rpoH*. The *rpoH* disruption mutant strain has poorer tolerance to ethanol, acetic acid, and temperature changes than the parental strain (Okamoto-Kainuma et al. 2011). Andrés-Barrao et al. (2012) used *A. pasteurianus* LMG 1262^T for oxidative fermentation in 3.9% ethanol and 0.1% acetic acid, and found that the expression of DnaK, GroES, and GroEL proteins were all upregulated in the late fermentation stage. In this study, compared with the 10-h samples, the expression level of *rpoH* (S101446_02833) continuously increased; at 88 h, *clpB* (S101446_02206) was upregulated but there were no significant changes to *dnaJ* (S101446_02334) or *dnaK* (S101446_02333), while *groES* (S101446_02983) and *groEL* (S101446_02984) were downregulated (Fig. 2 and Table SIII.9). The *recN* gene encodes a DNA repair protein (Sakurai et al. 2011). The *uvrABC* genes encode an excinuclease involved in DNA repair (Sakurai et al. 2011). The *dinB* gene encodes the damage-inducible, error-prone DNApolymerase IV (Sakurai et al. 2011). At 40 h, *recN* (S101446_01213), *uvrA* (S101446_02079), *uvrB* (S101446_02949), *uvrC* (S101446_01086), and *dinB* (S101446_01902) were significantly upregulated; at 64 h and 88 h, *uvrA* (S101446_02079) and *dinB* (S101446_01902) were significantly upregulated (Fig. 2 and Table SIII.9). Andrés-Barrao et al. (2012)

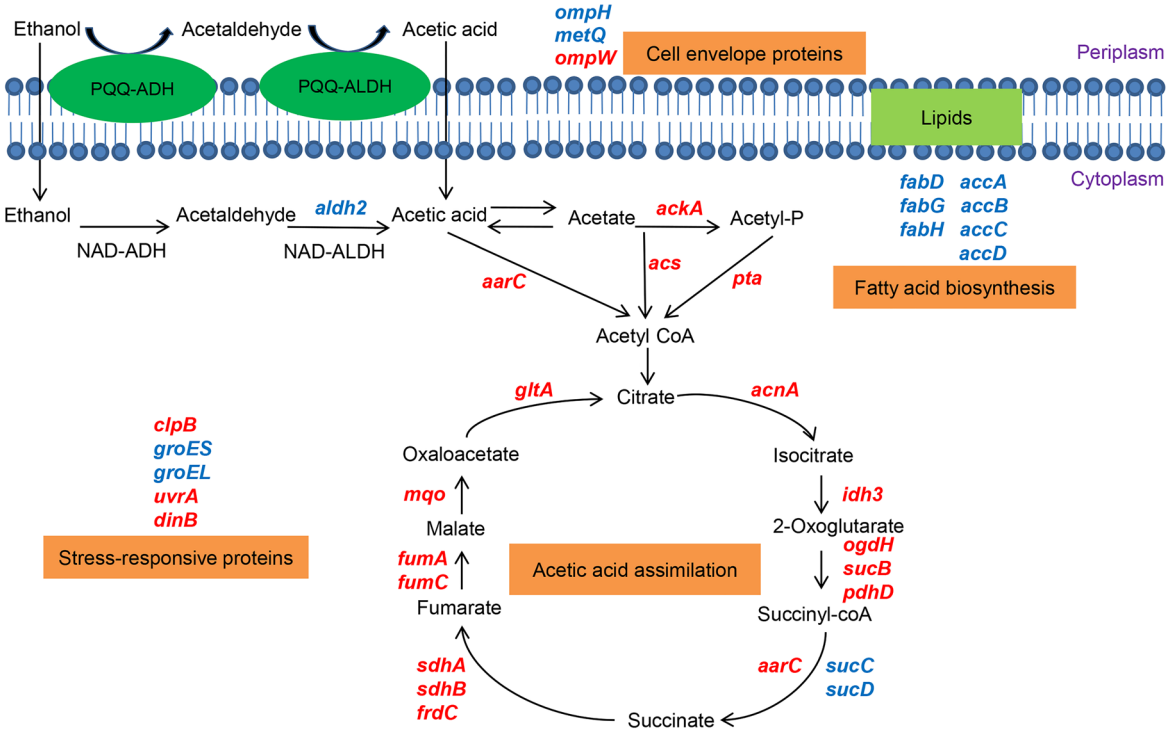

Fig. 3. The expression status of genes associated with acid resistance for *K. europaeus* CGMCC 20445 (acidity: 4.08 ± 0.21 g/100 ml). The upregulated and downregulated genes were denoted by red and blue texts, respectively. PQQ-ADH – pyrroloquinoline quinone-dependent alcohol dehydrogenase; PQQ-ALDH – pyrroloquinoline quinone-dependent aldehyde dehydrogenase; NAD-ADH – NAD-dependent alcohol dehydrogenase; NAD-ALDH – NAD-dependent acetaldehyde dehydrogenase.

used *Acetobacter pasteurianus* LMG 1262T for oxidative fermentation in 3.9% ethanol and 0.1% acetic acid. At the late stage of fermentation, the expression levels of *recN*, *uvrABC*, and *dinB* did not change significantly (Andrés-Barrao et al. 2012). It indicates differences in the expression levels of stress-responsive proteins between *A. pasteurianus* and *K. europaeus* during acetic acid fermentation, which may affect the difference in acid tolerance between *A. pasteurianus* and *K. europaeus*, and further research is needed.

Conclusions

Compared with the medium with the acidity of 1.60 ± 0.07 g/100 ml, the changes in the expression levels of the genes related to the acid tolerance mechanism of *K. europaeus* CGMCC 20445 in the medium with the acidity of 4.08 ± 0.21 g/100 ml are shown in Fig. 3. In this study, *K. europaeus* CGMCC 20445 mainly consumed intracellular acetic acid through acetic acid assimilation, similar to the acid resistance mechanism of *A. pasteurianus*. Acetic acid bacteria can regulate the expression of cell envelope proteins and the lipid content of the cell membrane, thereby regulating the concentration of ethanol and acetic acid in the cell. Acetic acid bacteria can also adjust the expression level of stress-responsive proteins to adapt to the gradual increase in acidity during acetic acid fermentation. The gene expression levels of cell envelope proteins and stress-responsive proteins differed between *K. europaeus* CGMCC 20445 and *A. pasteurianus* during acetic acid fermentation. These differences may lead to the increased tolerance of *K. europaeus* to higher concentrations of acetic acid than *A. pasteurianus*; a hypothesis that requires further study.

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Conflict of interest

The authors do not report any financial or personal connections with other persons or organizations, which might negatively affect the contents of this publication and/or claim authorship rights to this publication.

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