Food Microbiology 80 (2019) 77–84

Contents lists available at ScienceDirect

Food Microbiology

journal homepage: www.elsevier.com/locate/fm

Analysis of differential expression proteins reveals the key pathway in response to heat stress in *Alicyclobacillus acidoterrestris* DSM 3922^T

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ARTICLE INFO

Keywords:
Alicyclobacillus acidoterrestris
proteomics
Heat stress

ABSTRACT

For the purpose of investigating the heat resistance mechanism of *Alicyclobacillus acidoterrestris*, label-free quantification was used to reveal some cellular changes in *A. acidoterrestris* during heat stress. Totally, 545 differential expression proteins were respectively identified at heat stress of 65 °C for 5 min, of which 258 proteins were up-regulated and 287 proteins were down-regulated. These significantly changed proteins were mapped to 100 pathways and some of them were mostly related to protection or repair of macromolecules such as proteins and DNA, cell wall formation, which indicated that these proteins might play crucial roles in response to heat stress. The KEGG pathway analysis combined with protein functional analysis and further validation at mRNA level suggested that *A. acidoterrestris* sensed the temperature rise in environment through alterations in the secondary structure of DNA and RNA molecules. The biosynthesis of antibiotics pathway and the ribosomes might be involved in signal transduction in heat stress and further trigger a large number of proteins playing a critical role in the regulation of heat stress in *A. acidoterrestris*. The study firstly demonstrated the global physiological response to heat stress and the results provided a better understanding of thermal adaption mechanism of *A. acidoterrestris*.

1. Introduction

A. acidoterrestris, a thermo-acidophilic, strictly aerobic, heterotrophic, spore-forming and gram-positive bacterium, are capable of growing at pH values range from 3.0 to 6.0 and temperature range from 20 °C to 55 °C and can cause contamination in acidic beverages due to its heat and acid resistance (Yokota et al., 2007; Smit et al., 2011). The spores of *A. acidoterrestris* were much more resistant to heat compared with the growing cells (Bahçeci and Acar, 2007), which presented a serious challenge on the sterilization process of the juice industry. Since *Alicyclobacillus* was isolated in the 1980s (Deinhard et al., 1987), it has gained much research interests focusing on detection and control in fruits juice due to the particular acidophilic and thermophilic resistance (Mast et al., 2015; Cai et al., 2016). It is crucial to hazard control and effective utilization to investigate the molecular regulatory mechanism how *A. acidoterrestris* responded to heat and acid stress.

The stress response in bacteria involves a complex network that acts against the external stimulus and various bacteria mount appropriate adaptive mechanisms to survive diverse environmental changes (Filloux, 2012). Basically, it adapted challenging environment through regulating quantities and activities of intracellular enzyme to further adjust the expression of total protein in the cells (Bruijn, 2016). To study the stress reaction in bacteria would be of great benefit to illuminating the mechanism of resisting external severe environment and be instrumental in optimizing processing conditions to guarantee the microbial safety of food products (Bower and Daeschel, 1999).

The heat stress response had been investigated in numerous bacteria including *Escherichia coli*, *Bacillus subtilise*, *Bacillus cereus*, lactic acid bacteria and indicated that de novo protein synthesis was required for the survival at heat stress (Nonaka et al., 2006; Helmann et al., 2001; Periago et al., 2002; Van de Guchte et al., 2002). The research results showed that heat induced genes are involved in a variety of cellular processes, including protective processes, (post)transcriptional regulation, solute influx and efflux, and carbon metabolism (Petersohn et al., 2001; Price et al., 2001). In *A. acidoterrestris*, previous researches showed that the transcriptional levels of DnaK and DnaJ chaperonin were rapidly increased in a short time at heat and acid stress (Jiao et al., 2012, 2015), but the dynamic change of heat-induced proteins and the regulation mechanism of heat and acid adaptation have not been systematically studied. For the purpose of investigating the key regulator

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https://doi.org/10.1016/j.fm.2019.01.003
Received 7 July 2018; Received in revised form 12 November 2018; Accepted 6 January 2019

protein and elucidating the molecular regulatory mechanism of *A. acidoterrestris* at heat stress response, a Label-free quantification using liquid chromatography-tandem mass spectrometry (LC-ESI-MS/MS) were used to analyse differentially expressed proteins response to heat stress in *A. acidoterrestris* and RT-qPCR were used to further validate the functions of differential proteins, which would be beneficial for establishing a theoretical foundation for acidic beverages preservation and development of industrial strains for special purpose.

2. Materials and methods

2.1. Strain, media and culture conditions

2.1.1. Bacterial strain and culture medium

Alicyclobacillus acidoterrestris DSM 3922^T was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). According to the Jiao et al. (2015), AAM broth (*Alicyclobacillus acidoterrestris* medium) with pH 4.0 was used as the culture medium for *A. acidoterrestris*.

2.1.2. Growth and heat stress conditions

The strain of *A. acidoterrestris* was activated in AAM broth for 16 h at 45 °C, then the cell suspension were added into 50 mL fresh AAM medium in a 250 mL triangular flask with the inoculums concentration of 1%, and incubated in a thermostatic shaking bath (250 r/min) at 45 °C until the exponential phase. Five millilitres of bacterial cultures in logarithm growth period mentioned above were used as control and the other were heat-shocked in thermostatic shaking water baths at different heat treatment temperature of 60 °C, 65 °C, 70 °C for 5, 10, 15, 20, 25, 30 min. After heat stress, the bacterial viability and morphology of *A. acidoterrestris* were examined by plate counting method and scanning electron microscope (SEM) to check cell viability, respectively.

2.2. Observation of morphology of *A. acidoterrestris* with scanning electron microscope (SEM)

According to the method described by Harris (2002), bacteria samples washed five times with sterile ultrapure water were fixed with 2.5% glutaraldehyde, dehydrated with graded concentrations of alcohol, air dried and gold coated. The samples were then examined by SEM.

2.3. Total protein extraction and digestion

After heat stress, bacteria samples of control and treated groups were collected by centrifugation at 5000 × *g* for 10 min at 4 °C and then immediately frozen at −80 °C till protein extraction. The samples stored at −80 °C were washed twice with phosphate-buffered saline (PBS, pH 7.0), and suspended in cell lysis buffer (SDT) containing 4% w/v SDS, 100 mM Tris-HCl, 1 mM DTT, pH 7.6. After grind and ultrasonic lysis, the supernatants were collected and boiled for 5min. Filter-aided sample preparation (FASP) was used to convert the proteins into peptides as described by Wiśniewski et al. (2009). Fluorescence method (excitation wavelength 295 nm, emission wavelength 350 nm) were performed to quantify the total protein and peptide according to the method described by Wiśniewski et al. (2010).

2.4. LC-ESI-MS/MS analysis

Reverse phase-high performance liquid chromatography (RP-HPLC) separation was achieved on EASY-nLC1000 system at a flow rate of 300 nL/min with triplicate samples. The RP-HPLC mobile phases A and B were respectively 0.1% formic acid in water and 0.1% formic acid in acetonitrile. The mass spectrometry (MS) experiments were performed on a LTQ Orbitrap Velos Pro mass spectrometer (Thermo Finnigan, San Jose, CA) in data-dependent mode. A 180-min linear gradient elution was used, which consisted of three periods, 0–130 min (5%–40% B), 130–140 min (40%–90% B), 140–150 min (90% B) and 150–160 min (5% B).

The detection mode was set to positive ion and the spray voltage was 1.8 kV, the heated capillary was 250 °C and the scanning range was 350–1800 m/z. The 10 highest-intensity precursor ions were chosen from the full MS scan for CID (collision-induced dissociation) fragmentation and dynamic exclusion duration was 30 s. The resolution for full MS scan and subsequent MS/MS analyses were 70,000 and 17,500 at m/z 400, respectively. Three biological repetitions and two technical replicas were performed for each sample. All of the MS proteomics data have been deposited to iProX (http://www.iprox.org) and can be accessed with the accession IPX0001164000.

MaxQuant software (version 1.3.0.5, Max-Planck-Institute of Biochemistry, Am Klopferspitz, Germany) was used to analyse the MS data, which were searched against the UniProt database (uniprot_*Alicyclobacillus acidoterrestris* _4110_20160929.fasta), and the MaxQuant output files were subsequently uploaded into Perseus (Version 1.5.1.6) to calculate significance. According to the method described by Luber et al. (2010), protein abundance was calculated based on the normalized spectral protein intensity (label-free quantitation intensity, LFQ intensity) and label-free quantification analysis was performed using MaxQuant version 1.3.0.5. Proteins were defined as differentially expressed if the ratios were ≥1.5 or ≤0.67 compared with control with a significant change ($p < 0.05$) according to the criteria reported by Chen et al. (2016) and Le et al. (2013).

2.5. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analysis

The functional annotations of the differentially expressed proteins were performed using QuickGO (Binns et al., 2009). BLASTp searches used to find homologue sequences in the NCBI protein database and further analyses included Gene Ontology (GO) and Enzyme Code (EC) annotations. Subsequently, all the amino acid sequences of the identified proteins were submitted to the Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway database for analyzing that the differentially expressed proteins were mapped to metabolic pathways in KEGG (Kanehisa et al., 2012).

2.6. RNA extraction and validation by quantitative RT-qPCR analysis

Total RNA was isolated from *A. acidoterrestris* using RNAfast1000 (Pioneer Biotechnolgy, Inc) according to the instruction and then was reverse transcribed to cDNA using the RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Lithuania). Gene-specific qRT-PCR primers are listed in Table S1. Real-time RT-PCR was performed using a Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific, USA) and the TL988 Real-time Quantitative PCR Detection System (TIANLONG, Tianjin, China). The qRT-PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen (2001).

2.7. Statistical analysis

The significance of the differences between control and treatment groups was determined by a paired *t*-test using SPSS version 11.0. The level of significance was set at a *p*-value of less than 0.05 or 0.01.

3. Results

3.1. Effect of heat stress on cell viability and morphology of *A. acidoterrestris*

Three temperature levels exceeded the highest growth temperature

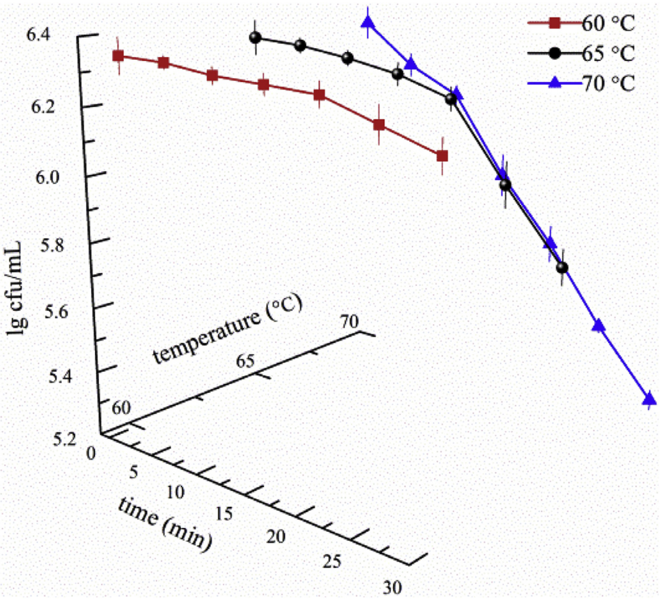

Fig. 1. Survival curves of *A. acidoterrestris* under heat stress.

of *A. acidoterrestris* were used for heat stress treatment. The data were analyzed by multivariate analysis of variance (MANOVA) and the results showed that the effect of treatment temperature and treatment time, the interaction of treatment temperature and treatment time on the survival of vegetative cells was extremely significant (the F values respectively are 444.45, 159.04, 40.92, $p < 0.01$). When *A. acidoterrestris* were heated at 60 °C, 65 °C and 70 °C for 5 min, the number of bacteria did not decrease significantly compared with the control group and when the treatment time was prolonged to 10 min, the bacterial counts of *A. acidoterrestris* decreased significantly ($p < 0.01$) (Fig. 1). To further check cell viability, cell morphology of *A. acidoterrestris* heat-treated at 60 °C, 65 °C, 70 °C for 5 min and 10 min were examined by scanning electron microscope (SEM). As shown in Fig. 2, the surface and edges of non-heat treated bacteria samples with the mellow and plump appearance are smooth and clear, showing a typical bacillus morphology, and cell morphology of *A. acidoterrestris* had no obvious change after heat treatment at 60 °C for 5min and 10 min. However, the surface of *A. acidoterrestris* became uneven after heat stress at 65 °C for 5 min, which indicated that bacterial cell wall was damaged to a certain degree. When *A. acidoterrestris* were respectively heated at 65 °C for 10 min, 70 °C for 5 min and 10 min, cell membrane damage were aggravated, cell shrinkage and membrane depression were observed in most cells. It can be inferred from the bacterial viability and the SEM examination results that *A. acidoterrestris* were alive and expression of intracellular protein might be changed after heat stress at 65 °C for 5 min. So, the samples heated at 65 °C for 5 min were selected to conduct the proteomics analysis.

3.2. LC-ESI-MS/MS analysis of differential expression proteins in *A. acidoterrestris* at heat stress

According to the LC-ESI-MS/MS results, the proteins with more than twice zero value in all the 6 LFQ analysis were excluded using perseus software and significant difference of protein expression was analyzed by a paired *t*-test (Table S2). Based on the fold change in protein expression, 545 differential expression proteins were respectively identified at heat stress for 5 min. Among the heat stress relevant proteins, 258 were observed to up-regulate their expression and 287 down-regulate their expression after heat stress of 65 °C for 5 min (Table S3).

3.3. Bioinformatics analysis of heat stress relevant differentially expressed proteins

Gene ontology (GO) analysis of the heat stress differentially expressed proteins showed that metabolic process, cellular process, single-organism process, developmental process, cellular component organization or biogenesis and response to stimulus were the top six biological processes that were relevant to heat stress (Fig. 3). Molecular function and cell component analysis reveals that the differential proteins were involved in structural molecule activity, catalytic activity, binding, translation regulation activity and protein binding, antioxidant activity, metallochaperone activity, signal transducer activity, molecular transducer activity and 83.6% of differential proteins were located in cell part (Fig. 3).

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the differentially expressed proteins showed that they were mapped into 100 pathways (Table S4), and the top ten pathway including ribosome, biosynthesis of antibiotics, metabolic pathway, purine metabolism, biosynthesis of secondary metabolites, alanine, aspartate and glutamate metabolism and so on were shown in Fig. 4

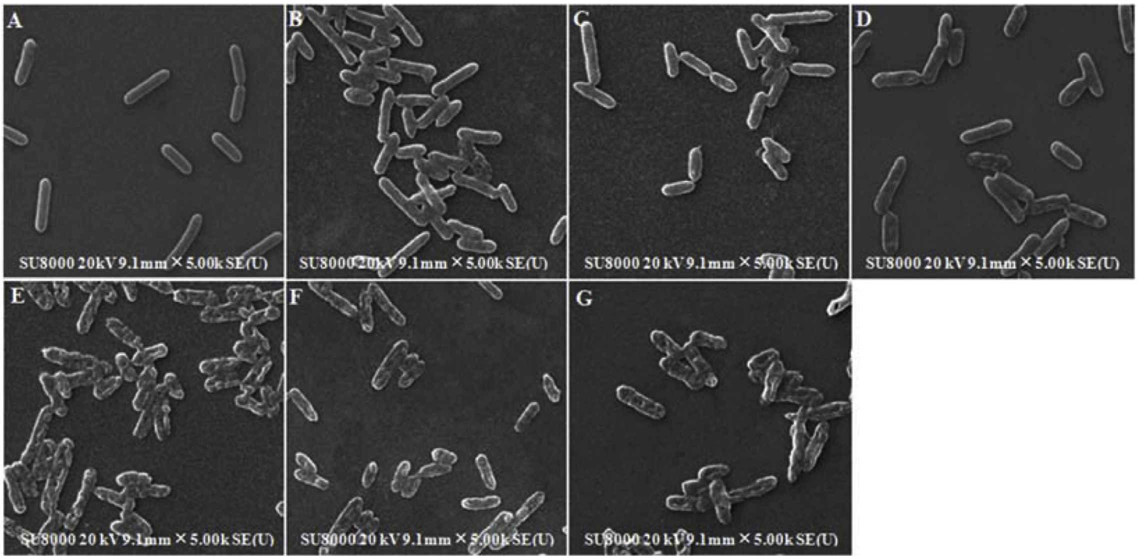

Fig. 2. The SEM photograph of morphology of *A. acidoterrestris.*
A. Control B—C. Heat stress at 60 °C for 5 min, 10 min D-E. Heat stress at 65 °C for 5 min, 10 min F-G. Heat stress at 70 °C for 5min, 10 min.

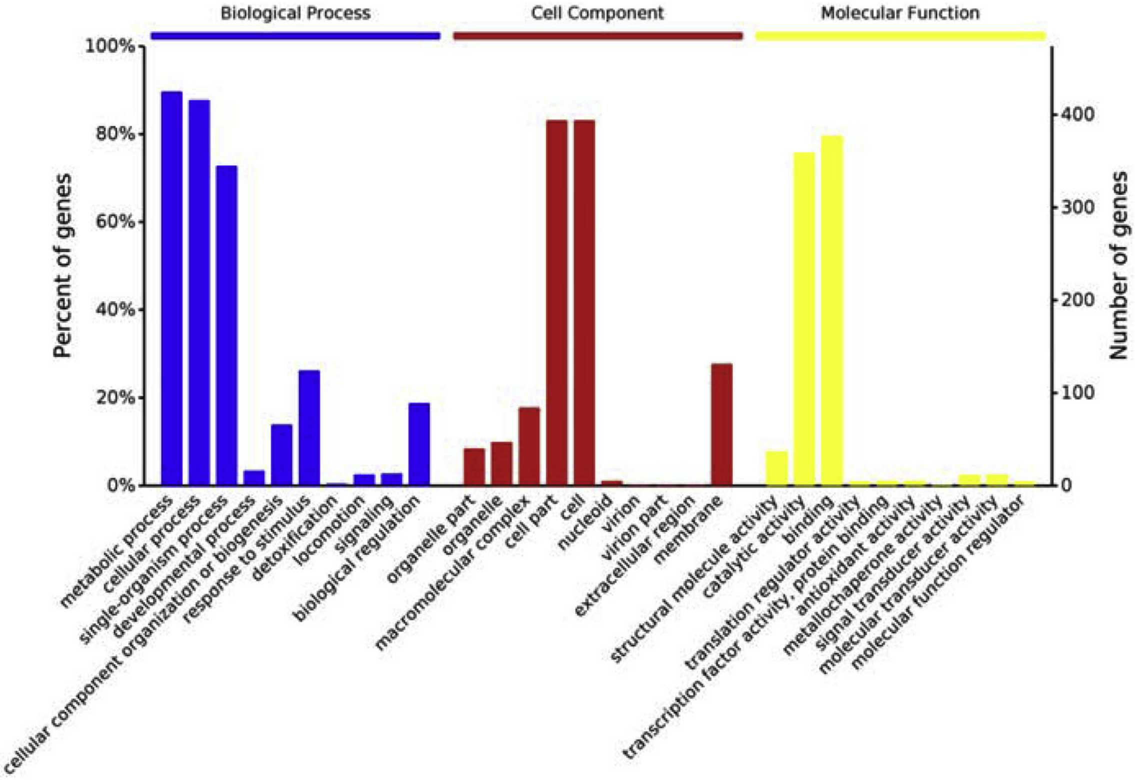

Fig. 3. Biological process, molecular function and cellular component analysis of differentially expressed proteins at heat stress.

($p < 0.05$). As shown in Table S4, thirty-five proteins were enriched in ribosome pathway and most of ribosomal subunits proteins were significantly up-regulated. Sixty-nine proteins were enriched in biosynthesis of antibiotics pathway and among them the proteins sharing aromatic-amino-acid aminotransferase activity and aspartokinase activity playing roles in the biosynthesis of leucin, tyrosine, phenylalanine, threonine and aspartate, which were the precursors for the synthesis of novobiocin and tabtoxinine-β-lactam. Accordingly, the alanine, aspartate and glutamate metabolism and biosynthesis of amino acids pathways were the two significant pathways. Other significantly changed proteins in biosynthesis of antibiotics pathway were important for the balance of metabolites in the pentose-phosphate pathway in relation to energy metabolism and the biosynthesis of precursor for aromatic-amino-acid, fatty acid, nucleotides and so on. Nine differential proteins were mapped into peptidoglycan biosynthesis and the differential proteins were involved in UDP-N-acetylmuramate–L-alanine ligase activity, D-alanine–D-alanine ligase activity, peptidoglycan glycosyltransferase activity, glutamate racemase activity, endolytic murein

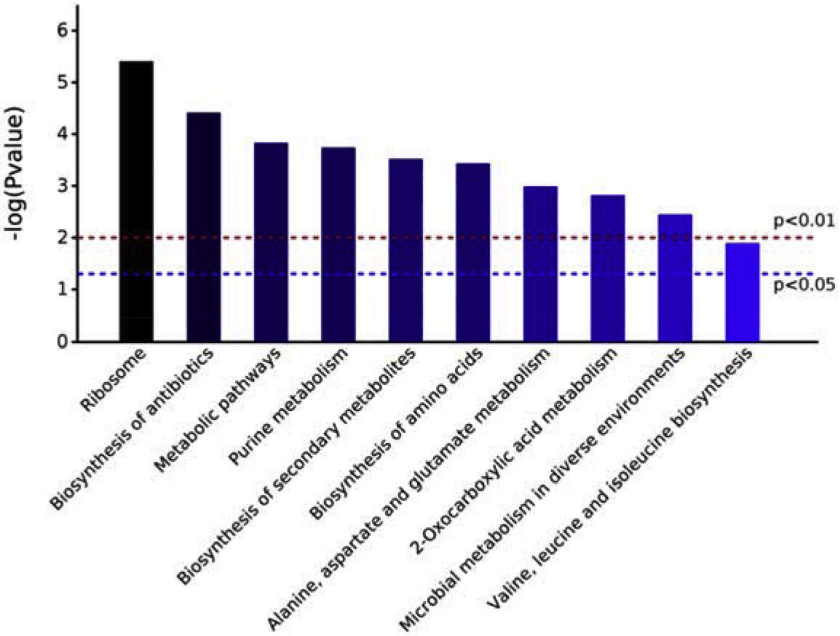

Fig. 4. The top ten enriched pathways of the differential proteins in KEGG.

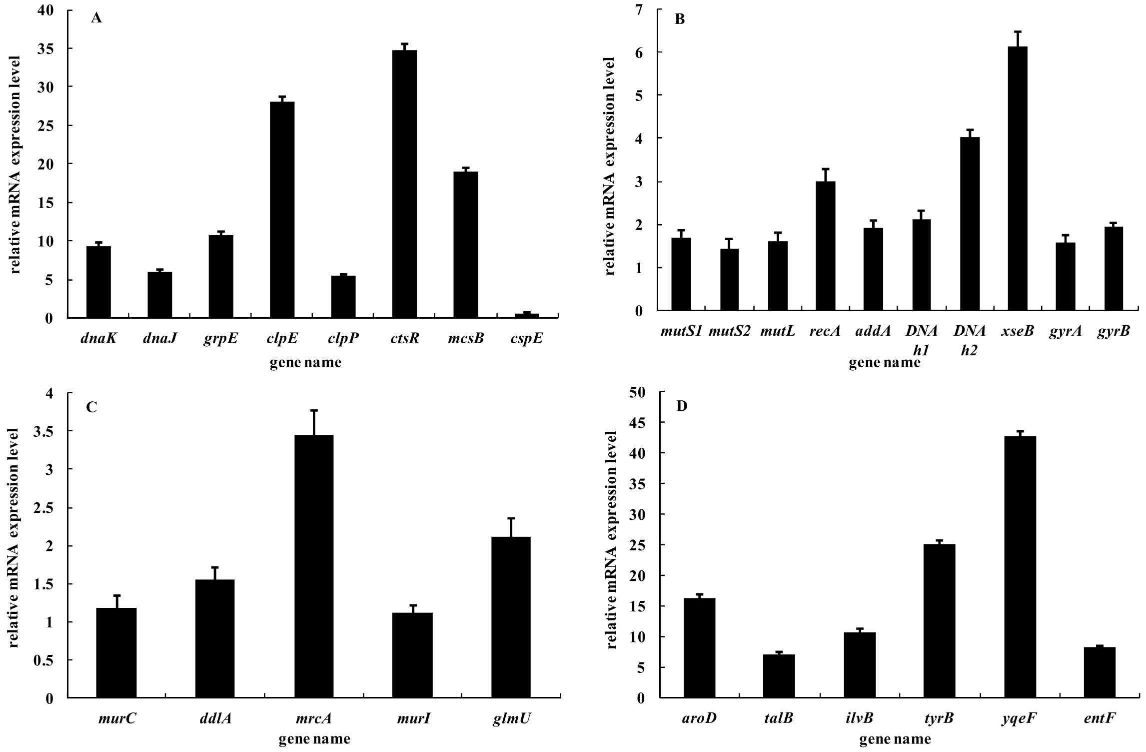

Fig. 5. Relative mRNA expression levels of the significantly changed proteins.
A. Heat-shock proteins B. DNA repair proteins C. Proteins related to cell wall biosynthesis D. Proteins related to antibiotic biosynthesis. The results represent the mean ± SD of three biological replicates.

transglycosylase, some proteins were acyl carrier protein and penicillin-binding protein and were closely related to the growing fatty acid chain, peptidoglycan synthesis and beta-Lactam resistance. Notably, seven proteins respectively encoded by mutL, mutS, recA, addA, xseB gene and other two DNA helicases were enriched in DNA mismatch repair and nucleotide excision repair pathway. The sharp increase of these proteins implied that DNA damage was induced by heat stress. How did the differential proteins in different pathways mentioned above function and interact with each other during heat stress is worthy of further discussion.

3.4. Validation of the significantly changed proteins at the mRNA level

To further confirm the results from LFQ proteomic analysis, RT-qPCR was performed to validate the predicted key heat-responsive protein genes which were involved in protein degradation and folding, repair of cell wall and DNA, and antibiotic biosynthesis based on he molecular function, pathway analysis and the reported gene function in the literatures, among which the transcription levels of 28 genes were up-regulated (Fig. 5). Of note, DnaK chaperonon system, Clp protease family, genes related to DNA repair, cell wall repair and antibiotic biosynthesis were the most significantly upregulated genes. Except for *grpE*, *clpP*, *xseB* and *yqeF* genes, the gene expression patterns were in accordance with the proteomic-level changes based on label-free quantitation intensity (Fig. 5; Table S3). The mRNA expression levels were higher than protein levels was may be attributed to the lag of protein expression behind mRNA, effects of post-transcriptional regulation and post-translational modification on the final protein expression, the differences in regulation mechanisms (such as synthesis and degradation rates) that act on both mRNA synthesis and protein synthesis, and these factors would ultimately affect the final protein expression amounts combined (Brockmann et al., 2007).

4. Discussion

Heat stress responses in bacteria involve a comprehensive network system of genes and proteins and it has been shown that de novo protein synthesis is required to increase thermotolerance (Periago et al., 2002). It is reported that DnaK family protein and proteases were closely related to heat stress and up-regulated during stress (Roncarati and Scarlato, 2017). In the present study, the expression level of *DnaK* and *DnaJ* genes were increased significantly in 5 min at both protein and mRNA level after the heat shock temperature of 65 °C (Table S3; Fig. 5A), which was consistent with previous results (Jiao et al., 2012, 2015) and suggested that they played an important role in response to heat stress in *A. acidoterrestris*. However, the expression of GrpE gene was contradictory in protein level and mRNA level (Table S3; Fig. 5A) and the inconsistency might be caused by the delay of protein expression after mRNA expression. It is now generally accepted that DnaK/DnaJ/GrpE chaperones minimize protein aggregation and protein disaggregation is accomplished by the chaperones belonging to the caseinolytic protease (Clp) family (Mishra and Grover, 2016). In the present study, the expression of clpE increased but the expression of clpP decreased. However, the mRNA expression of *clpE* and *clpP* gene enhanced significantly (Fig. 5A). The heat-shock response was a main cellular protection against adverse environment and the significant changes of DnaK family proteins and caseinolytic proteases indicated that heat-shock proteins were one of crucial survival and adaptation mechanisms for *A. acidoterrestris*.

What is worth mentioning is *cspE* was downregulated dramatically at heat stress of 65 °C, and it is consistent to the corresponding mRNA level (Fig. 5A). It is reported that *CspE* gene negatively regulated ***CspA*** gene which functions as a transcriptional activator of a subunit of DNA gyrase encoded by *gyrA* and *gyrB* genes (Bae et al., 1999; Wouters et al., 2000). In the present study, DNA gyrase subunit A and B were found dramatically increased at heat stress. It has been demonstrated that *CspE* expressed at high level can suppress the chromosome condensation, increase the level of plasmids DNA supercoiling which was critical for organisms' growth at low temperature (Bae et al., 2000; Sand et al., 2003). Although CspE is an early cold-inducible protein, the multiple molecule functions and significant changes during heat stress in the present study suggested that it might respond to heat stress in *A. acidoterrestris*.

Elevated temperature could change the structure of DNA and inactivate the enzymes related to DAN replication, which can lead to DNA damage and cell death (Purschke et al., 2010). Postreplicative mismatch repair (MMR) increases the fidelity of DNA replication and maintains genomic stability by correcting erroneous insertion, deletion, and mis-incorporation of bases in newly synthesized DNA (Fishel, 2015). The methyl-directed mismatch repair system including MutS, MutH and MutL are essential in detecting the mismatch and directing repair machinery to it (Mushayabasa and Bhunu, 2012). After heat stress of 65 °C for 5 min, a large number of enzymes in *A. acidoterrestris* related to DNA repair inclusive of MutS1 and MutS2, MutL, RecA, AddA and other two DNA helicases were up-regulated significantly and exodeoxyribonuclease VII small subunit encoded by xseB gene was down-regulated notably. MutS proteins recognize DNA mismatches and initiate mismatch repair (Tessmer et al., 2008). MutL mediates protein-protein interactions during DNA mismatch recognition, strand discrimination, strand removal and modulates the multiple functions of MutS and MutH (Guarné, 2012). Most MutL homologs have be shown to share endonuclease activity and interact to several single-strand exonucleases (Guarné, 2012). RecA together with RecBCD were required for repair of DNA breaks and homologous recombination (Amundsen et al., 2008), and it is reported that RecA and AddAB helicase-nuclease promote recombination-related DNA repair (Amundsen et al., 2008). The significant increase of RecA, AddA and two DNA helicases at heat stress might be involved in the recombination-related DNA repair in *A. acidoterrestris*. Exonuclease xseB gene of *meningococcal* was found to control an inducible DNA repair system (Morelle et al., 2005), the decease of xseB in the present study indicated that it might interact with MutL gene and be regulated by it.

Proteins related to peptidoglycan synthesis involving cell wall and outer membrane biogenesis such as UDP-N-acetylmuramate-L-alanine ligase encoded by *MurC* gene, D-Ala-D-Ala carboxypeptidase, and peptidoglycan glycosyltransferase encoded by *MrcA* gene, glutamate racemase encoded by *murI* gene, bifunctional protein GlmU changed significantly. Based on the SEM examination results (Fig. 2), the *A. acidoterrestris* cell wall was damaged after heat stress at 65 °C for 5 min. The damage of cell wall and cytoplasmic membrane of bacteria is believed to be critical events leading to death of bacteria. The monitoring and maintenance of cell envelope integrity is crucial for survival (Jordan et al., 2008) and repair of cytoplasmic membrane damage may involve the de novo synthesis of membrane components not just a simple resealing or reorganisation process. Chilton et al. (2001) found that repair of cytoplasmic membrane damage in *E. coli* necessitated peptidoglycan synthesis and inferred that cells attempted to synthesize cell wall material before repair of membrane damage was complete. The increase of peptidoglycan synthesis-associated enzymes indicated that *A. acidoterrestris* might protect cytoplasmic membrane from damage or repair cell membrane damage as soon as possible by regulating the synthesis of cell wall component.

Heat stress triggered a series of regulatory response and the interaction network between the transcriptional regulators and key heat-induced proteins remains not fully understood. Proteomic analysis of *A. acidoterrestris* under heat stress showed that differentially expressed proteins were mapped to 100 pathways (Table S4), which indicated that bacterial heat stress regulation was involved in a large and complex network system. Especially, the proteins associated with ribosomal subunit protein and ribosome assembly were significantly increased and the ribosome subunit formation and assembly was the most significant pathway based on the KEGG analysis. Vanbogelen and Neidhardt (1990) reported that the ribosome acted as a prokaryotic sensor for heat and cold shock response networks in *Escherichia coli*. The effect of temperature on the synthesis of proteins in bacteria was achieved by affecting the translational efficiency of ribosomes regulated by 'RNA thermometers' (Sengupta and Garrity, 2013). Our data reported here also provided an evidence for the theory.

At the same time, heat stress also led to a rapid adjustment of the bacterial physiology, among which metabolic pathway changes have been described in *Escherichia coli* and other bacteria during heat shock response (Ye et al., 2012; Fleury et al., 2009). It is reported that heat stress responses could trigger the expression of secondary metabolic genes and activate secondary metabolism by damaging the cell envelope (Yoon and Nodwell, 2014), which was consistent with our findings. In the present study, biosynthesis of secondary metabolites was one of the top ten significant KEGG pathways. Secondary metabolites are small, organic molecules including antibiotics, pigments, toxins and so on, which have diverse and often very potent biological activities (Yoon and Nodwell, 2014). Its synthesis can be induced by amino acids or other small molecules (Ruiz et al., 2010) and many secondary metabolites have been reported to perform important functions in the microbial interactions such quorum sensing, and the production of signaling molecules allows cells to communicate and respond to the environment in a coordinated way (Phelan et al., 2011). Notably, we found that biosynthesis of antibiotics pathway were the second significant pathways in response to heat stress. The expression of several proteins related to the biosynthesis of novobiocin and tabtoxinine-β-lactam were significantly increased in our study. The previous studies have shown that heat shock induced microorganisms to synthesisze antibiotics (Doull et al., 1993) and some microbial antibiotics including novobiocin, pefloxacin and chloramphenicol functioned regulatory role for DNA supercoiling (Cheung et al., 2003). Changes of DNA supercoiling might play a functional role in coupling stress signals to transcriptional activity (Sengupta and Garrity, 2013) and it was confirmed that increased negative supercoiling is necessary for proper elicitation of the *E. coli* osmotic shock response (Cheung et al., 2003). Novobiocin could inhibit the activity of DNA gyrase which negatively supercoiled closed circular double-stranded DNA to modulate DNA topology and maintain chromosomes in an underwound state (Papillon et al., 2013). Obviously, elevated temperature could result in changes in DNA supercoiling, which may then act as environmental sensors and trigger the further expression of heat stress-related genes (López-García and Forterre, 2000). Our data also showed that two type II topoisomerases, DNA gyrase subunit A and B, were sharply increased at protein and mRNA levels after heat stress for 5 min (Table S3; Fig. 5B) and eight differentially expressed proteins at heat stress respectively encoded by *mutS*, *mutL* etc. were mapped to the DNA mismatch repair pathway (Table S4), which might be induced by the changes of DNA conformation.

It is worth noting that alanine, aspartate and glutamate metabolism and biosynthesis of amino acids were in the top ten significant pathways. As reported, L-aspartate and L-tyrosine are the precursor of novobiocin and tabtoxinine-β-lactam (Calvert et al., 1973; Unkefer et al., 1987). Amino acids play a very important role as building block of proteins and there is considerable evidence that amino acid metabolism plays a crucial role in survival for a wide range of bacteria at heat stress (Lüders et al., 2009; Ye et al., 2012). It is reported that glutamate displayed an associated thermoprotective effect to allow protein disaggregation and refolding under high temperatures (Diamant et al., 2001) and arginine catabolism was closely related to energy production (Van de Guchte et al., 2002). As the precursor of monobactam and novobiocin biosynthesis, L-aspartate and L-tyrosine might act as signals and induce the synthesis of antibiotics based on the data reported here. So, our results indicated that the changes of DNA negative supercoiling caused by heat stress might induce the biosynthesis of antibiotics, which will provide new ideas for studying signal transduction and regulation network in *A. acidoterrestris* at heat stress.

It is well accepted that the most bactericidal antimicrobials could inhibit cell wall synthesis, DNA synthesis, RNA synthesis, or protein synthesis (Walsh, 2003) and increase the ability of bacteria to make biofilms (López et al., 2010). Peptidoglycan is an important constituent of the bacterial cell walls and the antibiotics can prevent its synthesis by affecting the enzyme transpeptidase and result in the inhibition of cell wall synthesis (Bhattacharjee, 2016). So, the synthesis of antibiotics at heat stress might activate both of β-lactam resistance and peptidoglycan biosynthesis pathway. Accordingly, *MrcA* gene related to negative regulation of cell lysis (Lin et al., 2011) and peptidoglycan biosynthesis-associated genes involved in the two pathways were changed dramatically in our study, which could serve as a strong evidence of the speculation above and indicated that the antibiotics synthesized at heat stress is key factors not only responding to conformational changes of DNA but also activating cell wall repair to protect cell membrane from damage. Previous studies showed that antibiotics triggered massive and complex changes in metabolism and global gene expression inclusive of the induction of specific stress response genes (Belenky et al., 2015; Brazas and Hancock, 2005; Dersch et al., 2017) and the result reported here added more evidence to the view.

5. Conclusion

Acidic fruit products spoilage caused by *A. acidotressis* and its spores has become an industry-wide problem. Based on the analysis of functions of differential proteins and involved pathways, our study demonstrated that physiological and biochemical response to heat stress in *A. acidotressis* is a complex and biological regulatory network. The DNA configuration changes at heat stress might function as biomolecule signal and induce activation of DNA gyrase and ribosome, thus trigger the biosynthesis of heat stress-responsive proteins and a series of intracellular dynamic changes including DNA repair, synthesis of specific antibiotics interacting with DNA gyrase and cell wall repair induced by antibiotics subsequently. Indeed, DNA gyrase subunit A and B were dramatically up-regulated at heat stress. The biosynthesis of antibiotics pathway may mediate and bridge the heat stress-associated biological processes and play critical role in regulating heat stress response of *A. acidotressis*. The analysis of key pathway response to heat stress provided a better understanding of thermal adaption mechanism in *A. acidotressis*, which contributed to its hazard control and effective utilization.

Ethical statement

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

Conflicts of interest

All the authors declare that they have no conflict of interest.

Acknowledgements

This work was supported by National Natural Science Foundation of China [grant numbers 31771949] and Program for Science and Technology Innovation Talents in Universities of Henan Province [grant numbers 17HASTIT037].

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.01.003.

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