J Ind Microbiol Biotechnol (2015) 42:601–616
DOI 10.1007/s10295-014-1579-0

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Society for Industrial Microbiology and Biotechnology

Comparative transcriptomic analysis reveals novel genes and regulatory mechanisms of *Tetragenococcus halophilus* in response to salt stress

Licui Liu · Lifang Si · Xin Meng · Lixin Luo

Received: 6 September 2014 / Accepted: 25 December 2014 / Published online: 8 January 2015

Abstract *Tetragenococcus halophilus*, a moderately halophilic Gram-positive bacterium, was isolated from Chinese style soy sauce. This species is a valuable resource for investigating salt tolerance mechanisms and improving salinity resistance in microorganisms. RNA-seq was used to sequence *T. halophilus* samples treated with 0 M (T1), 1 M (T2), and 3.5 M NaCl (T3). Comparative transcriptomic analyses of the different treatments were performed using gene ontology and Kyoto encyclopedia of genes and genome. The comparison of T1 and T2 by RNA-seq revealed that genes involved in transcription, translation, membrane system, and division were highly up-regulated under optimum salt condition. The comparison of T2 and T3 showed that genes related to heat shock proteins or the ATP-binding cassette transport systems were significantly up-regulated under maximum-salt condition. In addition, a considerable proportion of the significantly differently expressed genes identified in this study are novel. These data provide a crucial resource that may determine specific responses to salt stress in *T. halophilus*.

Keywords *Tetragenococcus halophilus* · Transcriptomic · Salt stress · RNA sequencing · Regulatory mechanisms

Electronic supplementary material The online version of this article (doi:10.1007/s10295-014-1579-0) contains supplementary material, which is available to authorized users.

L. Liu · L. Si · X. Meng · L. Luo (✉)
School of Bioscience and Bioengineering, South China University of Technology, Guangzhou 510006, China
e-mail: btlxluo@scut.edu.cn

Introduction

Salinity is one of the most important environmental stressors that severely impede microbial growth and development. Over the long course of evolution, halophilic bacteria can grow over a very broad range of salt concentrations. The capacity of these organisms to rapidly adjust to changes in the external salt concentration makes them potential candidates for bioprocessing as source of fermented food, compatible solutes, biomedical products, enzymes, polymers, cosmetics, and bioremediation [3, 33, 48]. In general, a sudden increase in the osmolarity of the environment is highly detrimental to the growth and survival of a microorganism because the environmental change triggers a rapid efflux of water from the cell to the outside medium; consequently, the cell experiences turgor pressure loss, intracellular solute concentration changes, and cell volume changes [60]. However, halophilic bacteria have developed two different adaptive strategies to cope with high salt concentration in the surrounding medium. Careful regulation of the intracellular ionic concentrations is a prerequisite, which is achieved by accumulating intracellular K^+ in concentrations higher than the external Na^+. This so-called "salt-in" strategy was first observed in *Halobacteriales* and the bacterium *Salinibacter ruber* [3, 16]. The widespread "low-salt-in" strategy is used by bacteria to balance the high ionic concentration in the medium via de novo biosynthesis or the uptake of organic compatible solutes [18]. Compatible solutes include amino acids, carbohydrates or their derivatives, sugars, and polyols (mainly glycerol and arabitol). These solutes have a stabilizing effect on the native structure of proteins and cell components both in vivo and in vitro. Compatible solutes can be amassed by the cell in exceedingly high concentrations without affecting cellular processes and the correct folding of proteins.

The term compatible solute can also be applied to solutes that protect macromolecules and cells against stresses such as high temperature, salinity, desiccation, and freezing [7, 27]. Halophilic bacteria have attracted increasing research interest since 1978, because of their adaptability to a wide range of salinity levels and their potential high commercial value; however, these microorganisms have constantly challenged scientists such that the complete understanding of their biology is hampered unresolved questions [18, 61].

Tetragenococcus halophilus (formerly known as *Pediococcus halophilus*) is a moderately halophilic Gram-positive lactic acid bacterium. This species is mainly used for brewing of soy sauce, a well-known condiment in Southeast Asia, China, and Japan. The genus *Tetragenococcus* was first described by British scientists through the reclassification of the halophilic lactic acid bacterium *Pediococcus halophilus* as *Tetragenococcus halophilus* [5]. *T. halophilus* grows during brine fermentation, where the salt concentration ranges from 12 to 26 %; it functions as a starter culture to promote the formation of the desired odor and improve the flavor of soy sauce [47]. In addition, *T. halophilus* plays an important role in other halophilic fermentation processes such as the production of soy paste, brined anchovies, fish sauce, Japanese fermented puffer fish ovaries, Indonesian "terasi" shrimp paste, and fermented mustard [26]. Similar to other genera of moderately halophilic Gram-positive bacteria (*Halobacillus* and *Salinicoccus*), *T. halophilus* is strictly chloride dependent and grows optimally in media containing 0.5–3.0 M NaCl. *T. halophilus* can tolerate high salt concentrations, which suggests that it possesses strong osmotic adjustment capacity to protect itself against deleterious hyperosmotic injury. Under adverse hypersaline stress, *T. halophilus* accumulates osmoprotectants by transporting carnitine and glycine betaine or by converting choline to glycine betaine [46]. Previous studies on the functions and expression of heat shock protein (HSP) encoding genes under conditions of high intracellular osmotic pressure demonstrated that GroESL, DnaK, and ClpB act as functional molecular chaperones and promote the adaptation of *T. halophilus* to salinity [54, 55]. However, as of this writing, a systematic and comprehensive study of salt tolerance response in *T. halophilus* is still not available.

The transcriptome has been defined as a collection of all the transcripts present in a given cell, which is evaluated qualitatively and quantitatively at a particular moment of cell development or during a specific physiological condition [66]. Transcript content, promoters, transcription start sites, open reading frames, regulatory noncoding regions, untranslated regions, and transcription units are all important aspects of the transcriptome that can provide valuable insight into the gene functions and molecular mechanisms that underlie molecular regulation in an organism at a specific time. Several researchers have already investigated and quantified the transcriptome of numerous bacteria via traditional approaches based on hybridization or tilling microarrays. Nevertheless, these techniques have several drawbacks, including their reliance on an existing genome sequence of the organism of interest, high background levels caused by nonspecific cross-hybridization, limited range of detection caused by the background and saturation of fluorescent signals, inability to detect transcripts with a low copy number per cell, and uncertain total coverage of the transcripts. Additionally, normalization methods and comparisons of expression data from different experiments are often difficult and arduous [9, 11].

RNA-seq provides researchers with a revolutionary method that is highly precise, rapid, reproducible, and cost-effective. This technique has been widely used in comparative transcriptomics to identify differential gene expression among various treatment conditions. Compared with eukaryotes, transcriptome-wide studies in bacteria have not been widely performed until recently owing to the simple microbial gene structures, as well as technical difficulties in the enrichment of mRNAs without poly-A tails. RNA-seq studies have revolutionized our current understanding of the complexity, plasticity, and regulation of bacterial transcriptomes [53]. In the present study, we generated a transcriptome dataset using the Illumina HiSeq™ 2500 platform to provide genetic information on the salt tolerance mechanisms of *T. halophilus*. We compared the transcriptomes of cells without salt stress, with optimum salt stress, and with high salt stress to determine changes in gene transcription, as well as identified the functions of the transcripts and KEGG pathways that showed transcriptional changes. The assembled, annotated transcriptome sequences and transcript abundance patterns provide valuable genetic resources for further investigations of the molecular mechanisms of salt tolerance in this species, and possibly in other halophilic bacteria. The salt tolerance of organisms can be improved by genetic engineering if the basic questions on the mechanisms of salt toxicity and defense response could be answered at the molecular level. Although the present study is a preliminary experiment, it contributes toward the identification and screening of more salt tolerance-related genes and factors to enhance salt resistance in other organisms, and possibly solve problems in food and environment industries [17, 24, 65].

Materials and methods

Strain and salt treatment

Tetragenococcus halophilus (CICC 10469), strain used in this study, was preserved by the China Center of Industrial Culture Collection (CICC). This strain was originally isolated from Chinese-type soy sauce and then identified via morphological,

physiological, biochemical, and 16S rDNA analyses in our laboratory [30]. *T. halophilus* CICC 10469 was grown at 30 °C in MRS medium (Difco). *T. halophilus* is chloride dependent, with an optimum NaCl concentration of approximately 1 M and an upper limit of approximately 3.5 M. Transcriptome analysis, bacterial cultivation, and salt-stress treatment were conducted as follows. Cells were first incubated at 30 °C in MRS liquid medium containing 1 M NaCl until the mid-logarithmic phase (27 h). Subsequently, 10-mL suspensions of fresh cells were collected by centrifugation at 8,000×*g* for 5 min and then inoculated into 50 mL MRS liquid medium with a final NaCl concentration of 0, 1, or 3.5 M in a 150-mL flask. For each treatment condition, three biological replicates were independently established. After 5 h of incubation, the cells were collected for RNA extraction.

RNA isolation

Total RNA of each sample was extracted with RNAiso™ plus (TaKaRa Bio Inc., Otsu, Shiga, Japan) and treated with RNase-free DNase I (Fermentas, Thermo Scientific, Waltham, USA) for 45 min at 37 °C to remove the residual DNA according to the manufacturer's instruction. The quantity of each RNA sample was checked using the NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE, USA). Meanwhile, RNA degradation and contamination were monitored on 1.2 % agarose gels. The integrity of the total RNA was assessed using Agilent 2200 Tape Station (Agilent Technologies, Santa Clara, CA, USA), and each sample had an RNA integrity number (RIN) of 9.9. To obtain complete gene expression information and reduce sequencing costs, a pooled RNA sample was obtained from the three replicates with the same salt treatment; equal amounts of mRNA were mixed after examination. The pooled RNA samples of each treatment condition were labeled T1 (0 M), T2 (1 M), and T3 (3.5 M).

cDNA library construction and sequencing

Sequencing libraries were generated using the Illumina Truseq™ RNA Sample Preparation Kit (Illumina Inc., San Diego, CA, USA) following the manufacturer's recommendations. Three index codes were added to attribute the sequences to the respective sample. The libraries were sequenced using the Illumina HiSeq™ 2500 platform, and 100-bp paired-end reads were generated.

RNA-seq: data analysis

Quality control

The raw data (raw reads) were first filtered through in-house Perl scripts to obtain high-quality clean data. This step was performed to discard reads containing the adapter, reads containing poly-N regions, and low-quality reads from raw data. Simultaneously, the Q20, Q30, and GC content levels of the clean data were evaluated. The average proportion of clean data reads in each sample was ~90.6 %, and all the downstream analyses were based on the clean data.

Reads mapping and gene annotation

The reference genome and gene model annotation files were directly downloaded from NCBI. The FM index [10] of the reference genome was built using Bowite (Broad Institute, Cambridge, MA, USA). The clean reads were then aligned to the reference genome using TopHat (Broad Institute, Cambridge, MA, USA). During the alignment of reads, seven mismatches and a gap length of 7 bp were allowed. The functional annotations of genetic variants were generated using ANNOVAR [64].

Quantification of gene expression level

The alignments reported by TopHat were processed by the Cufflinks software package to determine the differential expression of genes and transcripts between treatment conditions [57]. Expression values were measured in "fragments per kilobase of gene/transcript per million mapped reads" (FPKM). Data were visualized using the Integrated Genomic Viewer. Transcripts were quantified by assessing the total number of reads for the entire transcript using the program cuffdiff, part of the Cufflinks suite of tools for sequencing-based transcript assembly and quantification. The *P* values were adjusted using the Benjimini and Hochberg method [2]. Corrected *P* values ≤ 0.05 and the value of $|\log_2(\text{fold change})| \geq 1.5$ was set as the threshold to judge the significance of differences in gene expression between the treatment and control. Clustering of gene expression profiles was performed with Cluster and Java Treeview software. Hierarchical clustering was analyzed by the $\log_{10}$ (FPKM + 1) value for each significantly differentially expressed gene (SDEG).

Gene ontology (GO) and KEGG enrichment analyses of differentially expressed genes

GO enrichment analysis of each differentially expressed gene was performed by the Blast2GO program. We mapped all SDEGs to each term of the GO database (http://www.geneonology.org) and then calculated the gene number corresponding to each GO term. Using the hypergeometric test, we found significantly enriched GO terms compared with the genome background. The GO terms in SDEGs with corrected *P* values ≤ 0.05 were defined as significantly enriched. All SDEGs were assigned to the molecular

Table 1 Summary of RNA-seq results

Sample name	Raw reads	Clean reads	Clean bases (Gb)	Q20 (%)	Q30 (%)	GC content (%)
T1	16,550,964	14,989,572	1.50	95.18	91.98	39.5
T2	16,287,776	14,444,280	1.44	94.22	91.00	40.5
T3	16,926,786	15,652,774	1.57	96.39	93.36	40.0

function, biological process, and cellular component ontologies of the GO database. The Kyoto encyclopedia of genes and genome (KEGG) is a knowledge database and resource for the systematic analysis of high-level gene functions by linking higher order functional information, especially large-scale molecular datasets from genome sequencing and other high-throughout experimental technologies (http://www.genome.jp/kegg/). We used the KOBAS software (KOBAS, Surey, UK) to test the statistical enrichment of all SDEGs in the KEGG pathways.

Validation of RNA-seq data by q-PCR

To validate the RNA-seq data, the expression level of 10 randomly selected genes under the three different treatment conditions (0, 1, and 3.5 M) was analyzed by real-time quantitative PCR (q-PCR). The primers were designed using Primer Premier 5 software (PREMIER Biosoft, Palo Alto, CA, USA) and synthesized by Invitrogen. The primer pairs are summarized in Table S1. Salt treatment and RNA isolation were conducted as described above. The reverse-transcription reactions were performed using the PrimeScript RT reagent Kit (Takara, Da Lian, China), with approximately 1 μg total RNA in 20 μL reaction mixture. The q-PCR was performed by a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95 °C for 30 s, followed by 40 cycles of 94 °C for 5 s, and 60 °C for 34 s. Triplicate q-PCRs were performed for all reference and selected genes. A negative control was assigned to each reaction plate to monitor the experimental contamination. The expression levels of selected genes were normalized to that of 16S the rRNA gene, which was used as the internal reference gene. Relative gene expression levels were evaluated using the $2^{-\Delta\Delta Ct}$ method [31].

Results

To explore the molecular mechanisms of short-term salt tolerance in *T. halophilus*, we treated the cells in the logarithmic phase of growth with different NaCl concentrations (0, 1, and 3.5 M) for 5 h. We obtained high-quality pooled RNA samples from three biological replicates of each treatment condition. Three sequencing libraries were prepared from the T1, T2, T3 samples, and sequenced with the Illumina Hiseq™ 2500 platform.

Overview of RNA-seq results

A summary of the sequencing and assembly results is outlined in Table 1. A total of 16,550,964, 16,287,776, and 16,926,786 raw reads were generated for T1, T2, and T3, respectively (Sequence Read Archive accession number: SRP042175). After removal of the low-quality reads, which contained adapters and duplicate sequences or ambiguous bases, 90.57, 88.68, and 92.7 % of the raw data of T1, T2, and T3, respectively, remained as clean data. Among the clean reads, more than 91 % had Phred-like quality scores at the Q30 level (an error probability of 0.1 %). The total size of the clean nucleotides generated from each sample exceeded 1.4 Gb, that is, an unprecedented depth of 546 times (546-fold or 546× coverage) the genome size (~2.56 Mb). For the T1, T2, and T3 sample, 78.73, 79.79, and 79.21 % of the total reads from RNA-seq data, respectively, were uniquely mapped to the genome, whereas a smaller proportion was mapped to multiple loci in the genome.

Gene expression profile analysis

Based on the deep sequencing of the three libraries in this study, ~1,900 genes were detected in each library. The Venn diagram shows the distribution of expressed genes among the three samples (Fig. 1a). A total of 1,875 genes were co-expressed in all three samples. However, five, six, and five genes were NaCl concentration specific in T1, T2, and T3, respectively.

As previously mentioned, *T. halophilus* is chloride dependent, with an optimum NaCl concentration of 1 M. To conduct a broad survey of the transcriptomic responses to short-term salt stress in *T. halophilus*, three comparisons (T1 vs. T2, T3 vs. T2, and T1 vs. T3) were performed throughout the study. T2 was set as the control for the first two comparisons. The differences in gene expression among the different salt treatment conditions were calculated by the FPKM and Cufflinks software package. Corrected *P* values of 0.05 and values of $|\log_2(\text{fold change})| \geq 1.5$ were set as the thresholds for significant differential expression. The *P* values were adjusted by the Benjimini and Hochberg method. A total of 105 SDEGs were detected in the comparison of T1 and T2, with 71 up-regulated genes and 34 down-regulated genes. In the comparison between T3 and T2, a total of 112 SDEGs were detected, with 60 up-regulated genes and 52 down-regulated genes. The comparison of T1 and T3 detected a total

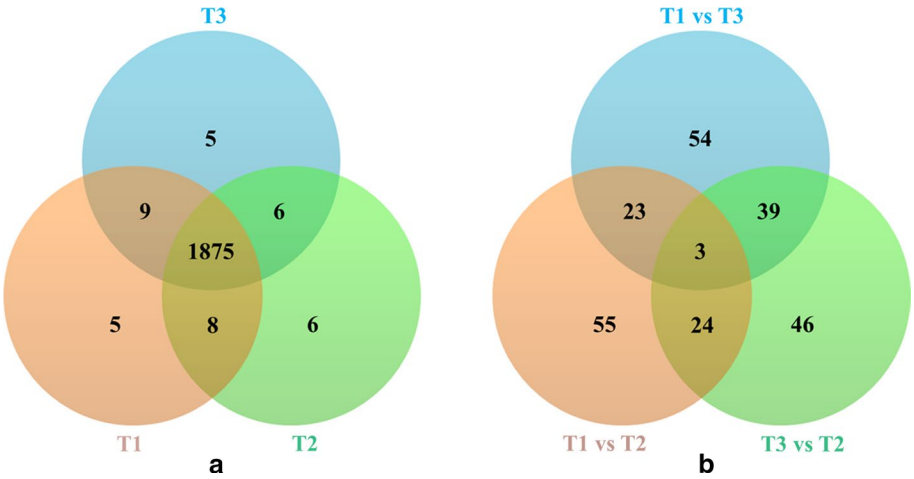

Fig. 1 Venn diagrams show the overlaps among the three samples and three comparisons. **a** Overlaps among the three samples; **b** overlapping SDEGs among the three comparisons

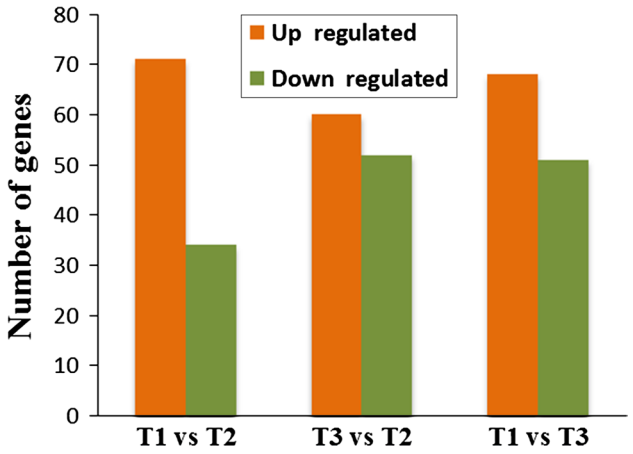

Fig. 2 Changes in gene expression profile in the three comparisons

of 119 SDEGs, with 68 up-regulated genes and 51 down-regulated genes (Fig. 2). Meanwhile, we independently performed hierarchical clustering of the SDEGs in three comparisons based on the $\log_{10}$ (FPKM + 1) values of the three treatments. Therefore, we could easily visualize the gene expression pattern (Fig. 3).

Experimental validation by q-PCR

The RNA-seq data were validated by q-PCR of 10 randomly selected genes. As shown in Fig. 4, q-PCR expression generally agreed with the changes in their transcript abundance as identified by RNA-seq, thereby suggesting that the RNA-seq data were reliable.

GO term and KEGG pathway enrichment analyses of SDEGs

The annotations of the SDEGs were analyzed using Blast2GO program base on the GO classification (http://www.geneontology.org). The annotations of all SDEGs were assigned to different functional categories (Dataset S1). A total of 77 SDEGs between T1 and T2 were categorized into 195 GO terms in the three main categories of the GO classification (cellular component, biological process, and molecular function). A total of 90 SDEGs between T3 and T2 were categorized into 222 GO terms, whereas 97 SDEGs between T1 and T3 were categorized into 198 GO terms. To understand the functions of SDEGs among the different salinity-stressed samples, further classification was conducted based on the correlation of GO terms (Fig. 5).

Cellular component category

In terms of the cellular components involved, the SDEGs between T1 and T2 were mainly mapped to the categories "cytoplasm" (GO:0005737), "integral to membrane, membrane, plasma membrane" (GO:0016021, GO:0016020, GO:0005886), and "ribosome" (GO:0005840). The SDEGs between T3 and T2 were mainly mapped to the categories "cytoplasm," "integral to membrane, membrane, plasma membrane," and "transcription factor complex" (GO:0005667). The SDEGs between T1 and T3 were mainly mapped to the categories "cytoplasm" and "integral to membrane, membrane, plasma membrane." These results suggested that the cytoplasm and membrane were dramatically affected during the salt treatment. Ribosomes were mainly affected when the NaCl concentration changed from low to optimum. However, the results did not reveal SDEGs belonging to the category "outer membrane-bounded periplasmic space" (GO:0030288) in the comparison. Similarly, the transcription factor complex was mainly affected when the NaCl concentration changed from optimum to maximum. In addition, when salt stress changed from the lower limit to the upper limit, the "integral to membrane, membrane, plasma membrane" categories were the dominant cellular component categories.

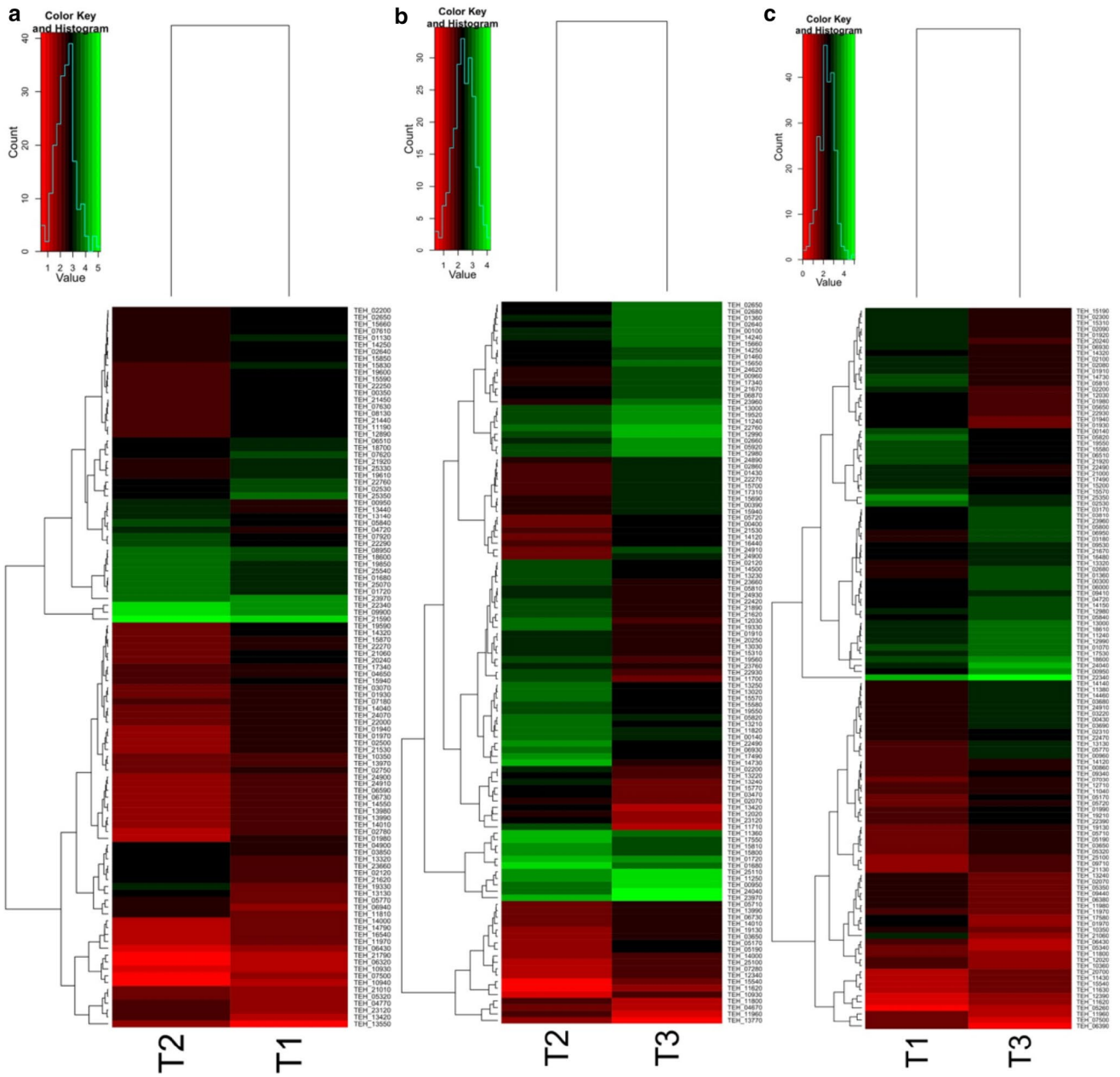

Fig. 3 Hierarchical clustering of the significantly differentially expressed gene in the three comparisons, using the RNA-seq data derived from three samples (T1, T2, and T3) based on $\log_{10}$ (FPKM + 1) values. We listed the corresponding SDEGs on the *right side* of the figure. The *red bands* identify low gene expression quantity, and the *green bands* represent the high gene expression quantity. **a** T1 versus T2; **b** T3 versus T2; **c** T1 versus T3

Biological process category

With respect to the biological processes involved, the SDEGs between T1 and T2 were mainly mapped to the categories "amino acid biosynthetic process or metabolic process," "DNA recombination, repair, replication, topological change, integration" (GO:0006310, GO:0006281, GO:0006260, GO:0006265, GO:0015074), "cell redox homeostasis and oxidation–reduction process" (GO:0045454, GO:0055114) and "carbohydrate metabolic process, gluconeogenesis and other glycometabolism" (GO:0005975, GO:0006094). The SDEGs between T3 and T2, as well as those between T1 and T3, were mainly mapped to the categories "amino acid biosynthetic process or metabolic process," "cell redox homeostasis and oxidation–reduction process," "carbohydrate metabolic process, gluconeogenesis and other glycometabolism,"

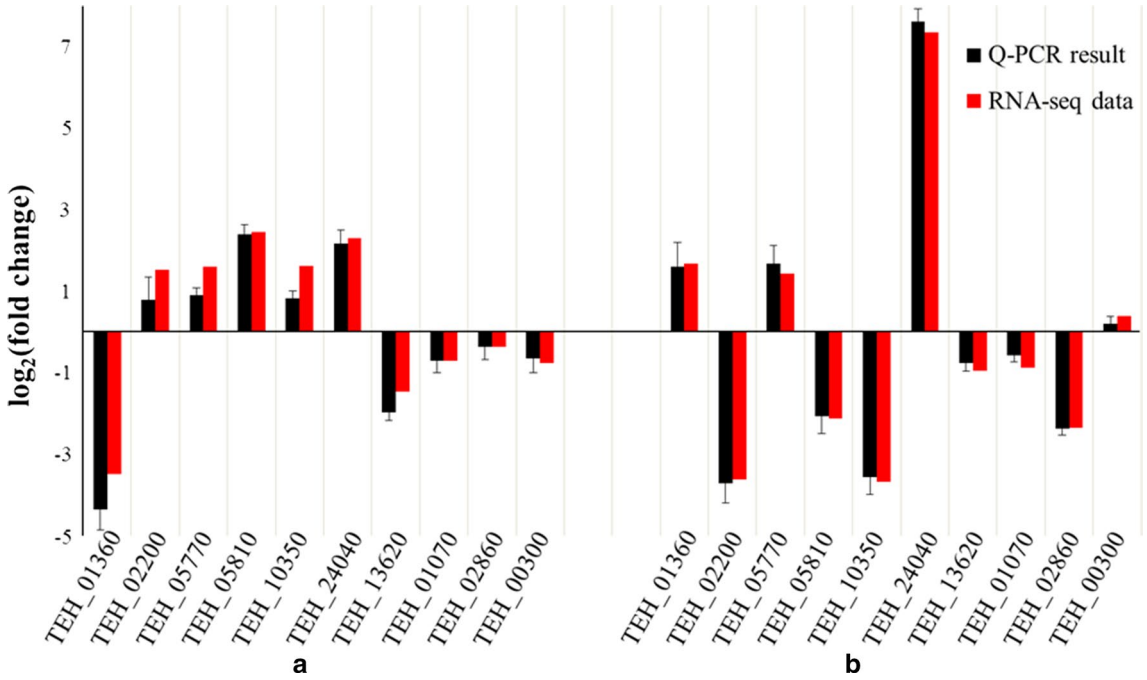

Fig. 4 Validation of RNA-seq data was performed by q-PCR. **a**, **b** The comparison "T1 versus T2" and "T3 versus T2", respectively. *Red bar* indicates transcript abundance changes calculated by the FPKM method. *Black bar* with associated standard error bar represents relative expression level determined by q-PCR using $2^{-\Delta\Delta Ct}$ method. Results represent mean standard deviations (±SD) of three experimental replicates. **a** The $\log_2$(fold change) values which from RNA-seq and q-PCR obeyed a linear correlation ($y = 0.9449x + 0.3616$, $R^2 = 0.9635$). **b** The $\log_2$(fold change) values which from RNA-seq and q-PCR obeyed a linear correlation ($y = 0.9793x - 0.0867$, $R^2 = 0.9977$)

and "transport" (GO:0006810). Although all the SDEGs of the three comparisons were mainly mapped to the "amino acid biosynthetic process or metabolic process" category, a wide variety of types and quantities of amino acids were involved. For instance, when salt stress changed from low to optimum, the amino acids tryptophan, tyrosine, proline, and arginine were mainly involved. However, when the salt stress changed from optimum to maximum, leucine, isoleucine, and valine were also involved, in addition to the aforementioned amino acids. When the salt stress changed from the lower limit to the upper limit, the amino acids glycine, cysteine, L-serine, and threonine were mainly involved. Therefore, the amino acids affected the microbial salt tolerance by a highly complex process. The terms "translation or regulation of translational fidelity" (GO:0006412, GO:0006450) and "ribosome biogenesis" (GO:0042254) were more active when the NaCl concentration changed from low to optimum compared with the other changes in salinity. In addition, "cell redox homeostasis," "oxidation–reduction process," "electron transport" (GO:0006118), "potassium, sodium, phosphate ion transport," "regulation of transcription, DNA-dependent" (GO:0006355), and "protein folding, protein targeting, protein refolding" (GO:0006457, GO:0006605, GO:0042026) were also dramatically affected during the course of the salt treatment.

Molecular function category

According to their molecular function, the SDEGs between T1 and T2 were mainly mapped to the categories "DNA binding, RNA binding" (GO:0003677, GO:0003723), "transferase activity" (GO:0016740), "ATP binding, ATPase activity" (GO:0005524, GO:0016887), and "structural constituent of ribosome" (GO:0003735). The SDEGs between T2 and T3 were mainly mapped to the categories "ATP binding, ATPase activity," "DNA binding, RNA binding," "transferase activity," "transporter activity" (GO:0005215), and "hydrolase activity" (GO:0016787). The SDEGs between T1 and T3 were mainly mapped to the categories "ATP binding, ATPase activity," "transferase activity," "transporter activity," and "oxidoreductase activity, acting on NADH or NADPH" (GO:0016616). In addition, other molecular functions were also dramatically affected during the course of salt treatment (not shown in Fig. 5), including "transmembrane transporter activity," "electron carrier activity," and "dehydrogenase activity."

KEGG pathway enrichment analyses

To identify the biological pathways that were active in the short-term salt-stress response of *T. halophilus*, we mapped

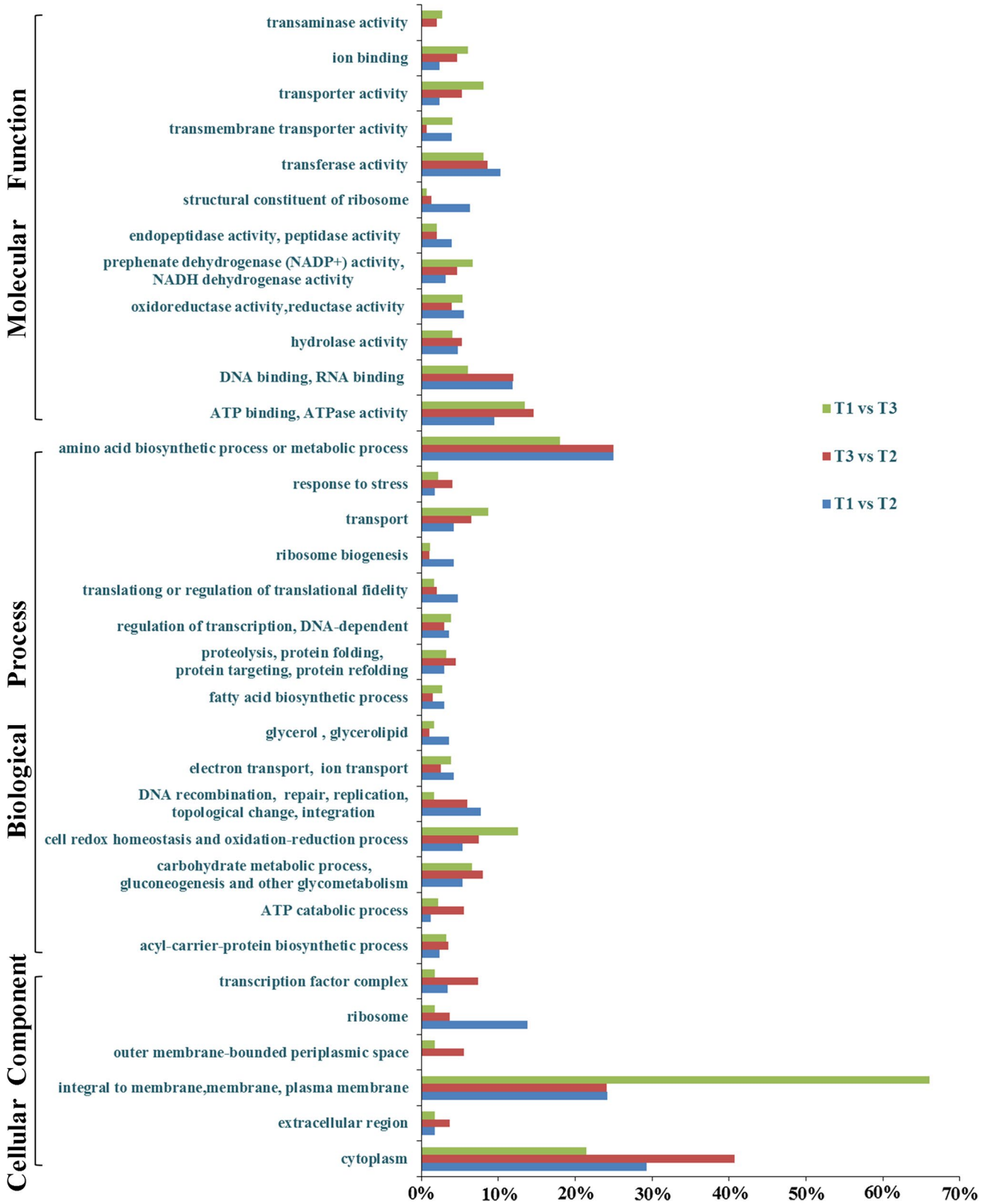

Fig. 5 Go function enrichment analysis of SDEGs among the different salinity-stressed samples

all SDEGs to reference canonical pathways in KEGG and compared them with the whole transcriptome background (Dataset S2). The SDEGs between T1 and T2 were assigned to 28 KEGG pathways. Those pathways with the greatest representation by unique genes were the metabolic pathways (thl01100) with 15 members, biosynthesis of secondary metabolites (thl01110) with 11 members, ribosome (thl03010) pathways with 7 members, and phenylalanine, tyrosine, and tryptophan biosynthesis (thl00400) with 6 members. The SDEGs between T2 and T3 were assigned to 31 KEGG pathways. The pathways with the greatest representation by unique genes were the metabolic pathways with 18 members, biosynthesis of secondary metabolites with 11 members, and ABC transporters (thl02010) with 6 members. The SDEGs between T1 and T3 were assigned to 39 KEGG pathways. The pathways with the greatest representation by unique genes were the metabolic pathways with 21 members, biosynthesis of secondary metabolites with 11 members, ABC transporters with 10 members, and microbial metabolism in diverse environments (thl01120) with 8 members. These annotations provided a valuable resource for investigating specific processes, functions, and pathways involved in short-term salt-stress response in *T. halophilus*.

To investigate the common SDEGs among the three comparisons, a Venn diagram was generated (Fig. 1b). A total of 27 SDEGs participated in the regulation of salt-stress response when the salt stress changed from low to optimum and from optimum to maximum. We surveyed the variations in their expression along the course of salt treatment (0, 1, and 3.5 M). Interestingly, for the three common SDEGs in all three comparisons, one (TEH_00950) was always up-regulated, one (TEH_02200) was always down-regulated, and one (TEH_24910) was first down-regulated and then up-regulated. Meanwhile, among the other 24 SDEGs, eight were first up-regulated and then down-regulated, whereas 16 were first down-regulated and then up-regulated; the expression levels of these genes were closer under T1 and T3. Therefore, these 24 SDEGs were significantly regulated when the cells grew under moderate salinity stress, but their levels became constant under limited salinity stress.

Based on the analyses of GO functions and KEGG pathways, 21 SDEGs were categorized into 74 GO terms and 9 SDEGs were assigned to 8 KEGG pathways. The regulations mainly involved categories "cytoplasm," "transcription factor complex," "amino acid (arginine, phenylalanine, tyrosine, and tryptophan) metabolism," "transport," and "glycolysis/gluconeogenesis." Meanwhile, the genes specifically expressed in each salt treatment (Fig. 1) were investigated. However, results demonstrated that a large proportion of these genes encoded hypothetical or putative proteins.

Discussion

Soy sauce is a traditional fermented condiment in Southeast Asia, especially in China. The two primary fermentation processes in China are low-salt solid-state fermentation and high-salt liquid-state fermentation. *T. halophilus* is used as a starter culture during brine fermentation and plays a pivotal role in improving the flavor of soy sauce under a wide range of salt stresses. Given our comparative transcriptome analysis, the differences in the expression of genes involved in the salt-stress response pathways were substantial at all the three salinity points investigated. Significant differences were noted in the molecular mechanisms by which *T. halophilus* adapts to the changing salinity environment. Consequently, the effects of the functional genes and pathways involved in the regulations of salinity tolerance should be understood.

Cell wall and membrane systems

The cell wall and membrane systems, which act as resistant barriers against environmental stress, suffer multiple forms of damage caused by salt-induced cellular dehydration [37]. During salt acclimation, the cell wall and membrane systems experience improved salt tolerance by changing their composition and the concentrations of unsaturated fatty acids, phospholipids, and peptidoglycans [3, 52, 63]. Our data showed that a large proportion of significantly differentially expressed transcripts were related to the cell wall and cell membrane according to GO classification and KEGG pathway enrichment analyses. For example, D-Alanine:D-Alanine ligase is closely involved in the biosynthesis of the peptidoglycan component of the bacterial cell wall. The encoded gene (*ddl2*) was up-regulated by 1.75-fold when the salinity changed from low to optimum, but this gene was down-regulated by 4.44-fold when the salinity changed from optimum to maximum. This change suggested that peptidoglycan might play a positive role in stress resistance when cells were under lower salt stress; cells can reduce the synthesis of peptidoglycan to promote ion transport across extra- and intracellular membranes when cells are under higher salinity conditions [4, 42]. Furthermore, the genes encoding 3-hydroxydecanoyl-ACP dehydratase (*fabN*), enoyl-ACP reductase (*fabI*), 3-oxoacyl-ACP synthase II (*fabF*), and beta-hydroxyacyl-ACP dehydratase (*fabZ*) were significantly regulated during the course of salinity treatment. These genes play important roles in fatty acid metabolism to maintain the membrane integrity in bacteria [15, 29].

Intercellular osmoprotectants

As mentioned above, several amino acids contributed to the osmoregulation in *T. halophilus* under different salt

treatments. For bacteria, amino acids generally act as "compatible solutes" to balance the difference in intracellular and extracellular osmotic pressure [67]. In particular, as a hydrophobic amino acid, the proline can act as a signaling molecule to influence defense pathways and relieve the osmotic stress caused by salt-induced dehydration. In the present study, the gene (*proC*) encoding pyrroline-5-carboxylate reductase (P5CR) and the gene (TEH_25060) encoding prolyl amino peptidase (PAP) were up-regulated during the course of salinity treatment. As the enzymes catalyzed the terminal reaction in proline biosynthesis, P5CR and PAP participate in the salt-stress response and show high expression, which promote the synthesis of proline for salt tolerance [8, 58, 62]. Glycine betaine has been widely identified as an osmoprotectant under various types of environmental stress [20, 43, 49]. The genes encoding the glycine betaine ABC transporter glycine betaine-binding protein (*opuAC*), permease protein (*opuAB*), ATP-binding protein (*busAA*), and permease/glycine betaine-binding protein (*busAB*) were all significantly up-regulated during the course of salinity treatment. Previous data suggested that glycine, serine, arginine, and other amino acids participate in the regulation of complex metabolic and developmental processes, and offer additional opportunities for organism improvement under adverse hyperosmotic stress [34, 39, 56]. Moreover, the accumulation of sucrose and other simple sugars also contributes to the stabilization of intercellular osmotic pressure [32]. In the present study, the genes encoding sucrose-specific IIC component (TEH_12190) and sucrose-specific IIBC component (*murP*) were significantly regulated under salinity stress. Both genes were involved in sucrose metabolism.

ABC transport system

The ABC transporter utilizes the energy of ATP binding and hydrolysis to transport various substrates across cellular membranes, including ions, amino acids, peptides, sugars, metabolic products, and other molecules that are mostly hydrophilic. Ion transport is a part of the ABC transport system that is known to play a crucial role in the osmoregulation. Generally, salt stress in combination with high pH can apparently induce greater injury because the high pH environment around a cell may cause metal ions and phosphorus to precipitate. Consequently, the absorption of minerals, such as the macro-elements K^+, Na^+, Cl^-, Ca^{2+}, and Mg^{2+}, as well as the micro-elements Fe^{2+}, Cu^{2+}, $H_2PO_4^-$, Zn^{2+}, and Mn^{2+} is affected, thereby disrupting ionic balance and pH homeostasis and inhibiting enzyme activity in cells [23, 38, 69]. The Na(+)/H(+) antiporters are membrane proteins that play a major role in the pH and Na^+ homeostasis of cells throughout the biological kingdom, from bacteria to humans and higher plants. Several Na(+)/H(+) antiporters are extremely sensitive to pH and regulated by an Na^+-specific regulatory network. NhaA is the key Na(+)/H(+) antiporter of *Escherichia coli* [40]. In the present study, the gene (TEH_11820) encoding a putative DASS family transporter, which is an Na^+ symporter [44], was significantly up-regulated by 2.48-fold when salinity changed from low to optimum, but this gene was down-regulated by 2.95-fold when salinity changed from optimum to maximum. The gene (TEH_12710) encoding a putative Na(+)/H(+) antiporter (with the GO function: potassium ion transport) was up-regulated by 2.15-fold when salinity changed from low to optimum. In addition, the gene (TEH_00440) encoding a putative ABC transporter ATP-binding protein and the gene (TEH_00450) encoding putative a ABC transporter permease protein were up-regulated by 2.71- and 2.77-fold, respectively, when salinity changed from low to optimum. According to KEGG, TEH_00440 and TEH_00450 are genes involved in the zinc transport system. In general, two acidic pH resistance response systems have been clearly defined in enterobacteria or *Escherichia coli*, namely, the inducible decarboxylase system and acid shock protein (ASP) system [1]. The inducible decarboxylase system includes three acid resistance systems: glutamate-dependent system (consists of two glutamate decarboxylase isoforms: GadA and GadB, as well as an integral membrane protein GadC), arginine-dependent system, and oxidative systems [13]. The ASP system is controlled by four regulatory proteins: the iron regulatory protein Fur (ferric uptake regulation), two-component regulatory system PhoPQ (senses magnesium and protons), alternative RNA polymerase sigma factor σ^S (RpoS; encoded by the *rpoS* gene), and classic response regulator OmpR [12]. ASPs prevent or repair acidic pH-induced damage to macromolecules. The Fur protein exerts control over a series of genes to maintain the intracellular and extracellular iron balance, meanwhile, the Fur regulator acts in the positive control of several RpoS-independent ASPs [14, 19]. Three genes encode a "putative Fur family transcriptional regulator" in *T. halophilus*: TEH_13470, TEH_17000, and TEH_20140; these genes were up-regulated by 2.76-, 1.76-, and 2.16-fold, respectively, when the salinity changed from low to optimum. However, they were down-regulated by 2.67-, 2.52-, and 3.11-fold when salinity changed from optimum to maximum, respectively. RpoS is known to be a critical regulator of the stationary and exponential phase in cells subjected to acid stress [1, 12]. RpoS belongs to a protein family containing homologs of the major housekeeping sigma factor RpoD (σ^{70}). Both RpoS and RpoD share structural homology. In the regulation of acid tolerance, RpoD can partially replace the RpoS subunit in binding to the bacterial RNA polymerase core enzyme and interacting with the promoter region to initiate transcription [68]. In the present study, the *rpoD* gene

was down-regulated by 2.39-fold when salinity changed from low to optimum, but was up-regulated by 2.10-fold when salinity changed from optimum to maximum. Consequently, these genes function in the ABC transport system to adjust the osmotic pressure induced by salinity stress. ABC transporters act as members of a protein superfamily that is present in all three domains of life. These proteins transport a wide variety of substrates across extra- and intracellular membranes. Therefore, these transporters have various regulatory functions for salt tolerance, rather than simply acting as ion transporters.

Central carbon metabolism (CCM)

The term CCM describes the integration of transport pathways and oxidation of main carbon sources inside the cell. In most bacteria, the main CCM pathways are the phosphotransferase system, glycolysis, gluconeogenesis, glutaminolysis, pentose phosphate pathway, and tricarboxylic acid cycle with the glyoxylate bypass [41]. CCM plays an important role in salt-induced regulations in cells. First, CCM generates energy to maintain energy-demanding osmoregulation, which includes ion transport, protein transport, redox balance, antioxidation, and other ATP-dependent processes. Second, CCM provides intermediates to fatty acid biosynthesis, amino acid biosynthesis, and nucleotide biosynthesis. In our study, a large number of significantly differentially expressed transcripts were related to CCM. For instance, the genes encoding 6-phosphofructokinase (*pfk*), 6-phospho-β-glucosidase (*bglA*), and putative trehalose 6-phosphate phosphorylase (TEH_00400) were significantly regulated under salt stress. Therefore, *T. halophilus* may change the direction of its main metabolic flow to enhance its osmotic adjustment capacity against salt-induced injury. In addition, some regulators of phosphate-associated genes were reported to be induced by salt stress. The accumulation of phosphate enhances tolerance to salt stress in *Arabidopsis* [35], and the phosphate transport system is highly activated by NaCl in *Deinococcus radiodurans* [21].

Main regulatory mechanisms of *T. halophilus* in response to salinity stress

To investigate the main regulatory mechanisms of *T. halophilus* in response to salinity stress, the significantly up-regulated genes were analyzed (Tables 2, 3, with the corresponding GO and KEGG enrichment analyses in the supplementary materials). In the present study, comparative transcriptomic analysis of samples treated with 0- and 1-M NaCl indicated that the up-regulated genes in *T. halophilus* grown in optimum salinity conditions were mainly related to transcription and translation. These genes encoded ribosomal proteins and structural constituents of ribosomes or were involved in ribosome biogenesis, ribokinase activity, and tRNA-dihydrouridine synthesis. Other genes were related to the membrane system and cell division, such as glycerolipid metabolism or lipid A and fatty acid biosynthesis. Therefore, the up-regulation of genes related to transcription, translation, membrane systems, and cell division could provide necessary conditions for cell growth in 1 M salinity. Simultaneously, comparative transcriptomic analysis of samples treated with 1 and 3.5 M NaCl suggested that when *T. halophilus* was grown under maximum salinity, the up-regulated genes were mainly related to heat shock proteins (HSPs), such as the genes *grpE, groES, clpE, hslV, hslU, hrcA, dnaK,* and *groEL*, or related to the ABC transport system. HSPs have essential roles in protein synthesis, transport, folding, repair, and turnover; these proteins are often referred to as molecular chaperones. In prokaryotes, the major HSPs are encoded by Class I genes that are constitutively expressed at all temperatures. Class I genes encode two major chaperone proteins: the 70-kDa HrcA-GrpE-DnaK-DnaJ and 60-kDa GroEL-GroES. Following a temperature upshift, the expression of these genes abruptly accelerates and is negatively regulated by a repressor encoded by *hrcA* [50]. The HrcA repressor exerts its function interacting with a cis-active inverted repeat called CIRCE [71]. The genes encoding the heat-inducible ATP-dependent protease HslVU like *hslV* (also called *clpQ*) and *hslU* (also called *clpX*) belong to another class of heat shock genes; these genes are expressed from a vegetative promoter P_A and do not contain the CICRE element [70]. The heat shock response is a general homeostatic mechanism that enables animal, plant, and bacterial cells to survive various stress conditions [36]. The induction of HSPs during salt stress has been confirmed in *Lactococcus lactis* [28], *Bacillus subtilis* [59], and other organisms. Furthermore, several up-regulated genes were rare or lacked definitive evidence of induction by salt stress; examples are the acyl carrier protein-encoding gene *acpP*, TEH_21670 and TEH_03650 (which both encode putative aldo-keto reductases), arginine deiminase-encoding gene *arcA*, TEH_14240 and TEH_24620 (which both encode aldehyde dehydrogenases), TEH_00400 (which encodes a putative trehalose 6-phosphate phosphorylase). Further study is necessary to determine how these genes affect the osmotic adjustment capacity of *T. halophilus*. In addition, a considerable proportion of the SDEGs identified in this study are novel. Among the 244 total SDEGs, 52 could not be mapped to any GO category. Many of these genes exhibited a substantial change in expression levels during the course of salt-stress treatment, including TEH_00960, TEH_01430, TEH_01460, TEH_01680, TEH_01970, and others. Meanwhile, we found that the gene without functional information (i.e., putative and hypothetical genes)

Table 2 In the comparison of T1 and T2, significantly up-regulated genes of the *T. halophilus* under 1 M NaCl condition

Locus tag	Gene name	Fold change	Product
TEH_19330	*acpP*	9.88	Acyl carrier protein
TEH_13130		9.79	Hypothetical protein
TEH_13420		8.88	Hypothetical protein
TEH_13550		8.05	Hypothetical protein
TEH_06940	*rpmG*	7.97	50S ribosomal protein L33
TEH_23120		7.62	Hypothetical protein
TEH_22340	*rpmB*	6.06	50S ribosomal protein L28
TEH_21620		5.54	Hypothetical protein
TEH_23660		4.82	Putative replication protein
TEH_02120		4.58	Hypothetical protein
TEH_05770		4.58	Hypothetical protein
TEH_11810	*aroQ*	4.54	Chorismate mutase
TEH_05840	*dtpT*	4.33	Putative di-/tripeptide transporter
TEH_25540	*rpmH*	4.29	50S ribosomal protein L34
TEH_04720	*rbsK*	4.24	Ribokinase
TEH_22290	*acpP*	4.13	Acyl carrier protein
TEH_05320		4.12	Putative nicotinamide mononucleotide
TEH_21010		3.91	Hypothetical protei
TEH_07920		3.78	Citrate transporter
TEH_03850		3.70	Hypothetical protein
TEH_13440		3.60	Hypothetical protein
TEH_04770		3.52	Hypothetical protein
TEH_19850		3.50	Hypothetical protei
TEH_01680		3.26	Hypothetical protein
TEH_08950		3.24	Hypothetical protein
TEH_25070	*rpsI*	3.21	30S ribosomal protein S9
TEH_13140		3.19	Hypothetical protein
TEH_13320	*fabN*	3.18	3-hydroxydecanoyl-[acyl-carrier-protein]dehydratase FabN
TEH_09900	*rpmE2*	3.16	50S ribosomal protein L31 type B
TEH_04900		3.14	Putative tRNA-dihydrouridine synthase
TEH_00950		3.12	Putative C56 family peptidase
TEH_01720	*rplY*	3.09	50S ribosomal protein L25
TEH_21590		3.04	Hypothetical protein
TEH_18600	*opuAC*	2.99	Glycine betaine ABC transporter glycine betaine-binding protein

occupied a large proportion among the SDEGs, namely, 47.06 and 46.67 %, as shown in Tables 2 and 3, respectively. These unmapped or uncertain transcripts are a crucial resource that may determine specific responses to salt stress in *T. halophilus*.

Conclusions and outlook

To the best of our knowledge, this study is the first to report the Illumina sequencing of the *T. halophilus* transcriptome under stress acclimation. Approximately 4.51 Gb of clean data was generated. Based on deep sequencing of the three libraries in this study, ~1,900 genes were detected for each library. We used the FPKM to normalize the expression level of genes and identified genes with significant changes in their expression among the samples. BLAST analysis of all SDEGs in public databases (GO and KEGG) allowed us to obtain their functional annotations and classifications, from which we analyzed the regulatory mechanisms of *T. halophilus* in response to salt stress. The complexity of the regulation of salt tolerance involved in enormous biological processes was demonstrated by our data. We highlighted the link between the main gene functions and salt tolerance regulatory mechanisms. The large number of transcriptomic sequences and their functional annotations will provide sufficient resources for future molecular studies of *T. halophilus* and other halophilic bacteria. Despite the

Table 3 In the comparison of T2 and T3, significantly up-regulated genes of the *T. halophilus* under 3.5 M NaCl condition

Locus tag	Gene name	Fold change	Product
TEH_05170		17.32	Putative ABC transporter ATP-binding protein
TEH_24040		15.41	Hypothetical protein
TEH_24910	*arcA*	13.59	Arginine deiminase
TEH_05190		11.94	Putative ABC transporter permease protein
TEH_15540	*glgB*	11.80	1,4-Alpha-glucan branching enzyme
TEH_10930	*bglA*	9.33	6-Phospho-beta-glucosidase
TEH_23960		8.47	Uracil-DNA glycosylase family protein
TEH_24900	*arcB*	8.23	Ornithine carbamoyltransferase
TEH_11620	*idh*	7.96	Myo-inositol 2-dehydrogenase
TEH_25100		7.54	Peptidase A24 family protein
TEH_03650		7.40	Putative aldo-keto reductase
TEH_02660	*gpo*	6.57	Glutathione peroxidase
TEH_12990	*grpE*	6.22	GrpE protein
TEH_19130		6.11	Putative ABC transporter substrate-binding protein
TEH_14120		5.99	Hypothetical protein
TEH_17340		5.72	Hypothetical protein
TEH_15660	*hslV*	5.60	ATP-dependent protease HslV
TEH_14240		5.55	Aldehyde dehydrogenase
TEH_17310		5.29	Putative ABC transporter substrate-binding protein
TEH_05710	*ilvE*	5.17	Branched-chain amino acid aminotransferase
TEH_02650		5.13	Putative MarR family transcriptional regulator
TEH_02680		4.98	Hypothetical protein
TEH_15700	*rnhB*	4.92	Ribonuclease HII
TEH_21530	*dtd*	4.90	D-tyrosyl-tRNA(Tyr) deacylase
TEH_07280		4.79	Putative acetyltransferase
TEH_00400		4.55	Putative trehalose 6-phosphate phosphorylase
TEH_01360		4.55	Putative tetr family transcriptional regulator
TEH_02640	*trxB*	4.48	Thioredoxin reductase
TEH_12340		4.38	Putative ABC transporter substrate-binding protein
TEH_15690	*dprA*	4.37	DNA processing protein
TEH_00960		4.32	Hypothetical protein
TEH_06730	*sbcC*	4.27	Nuclease SbcCD subunit C
TEH_24620		4.23	Putative aldehyde dehydrogenase
TEH_14010	*aroA*	4.22	3-phosphoshikimate 1-carboxyvinyltransferase
TEH_05720		4.22	L-2-hydroxyisocaproate dehydrogenase
TEH_11240	*groES*	4.22	10 kDa chaperonin
TEH_13990	*aroC*	4.21	Chorismate synthase
TEH_02860	*ntpI*	4.17	V-type Na(+)-transporting ATPase subunit I
TEH_00950		4.03	Putative C56 family peptidase
TEH_22270	*smc*	4.03	Chromosome partition protein SMC
TEH_21670		4.00	Putative aldo-keto reductase
TEH_00390	*pgmB*	3.97	Beta-phosphoglucomutase/glucose-1-phosphate phosphodismutase phosphodismutase
TEH_24890	*arcC*	3.97	Carbamate kinase
TEH_14000	*tyrA*	3.96	Prephenate dehydrogenase
TEH_19520	*clpE*	3.93	ATP-dependent Clp protease ATP-binding subunit ClpE
TEH_15650	*hslU*	3.87	ATP-dependent Hsl protease ATP-binding subunit HslU
TEH_12980	*hrcA*	3.77	Heat-inducible transcriptional repressor HrcA
TEH_01430		3.66	Hypothetical protein

Table 3 continued

Locus tag	Gene name	Fold change	Product
TEH_01460		3.64	Hypothetical protein
TEH_13000	*dnaK*	3.63	Chaperone protein DnaK
TEH_15940		3.55	Putative ABC transporter ATP-binding protein
TEH_23970		3.54	Hypothetical protein
TEH_14250		3.53	Hypothetical protein
TEH_06870		3.47	Putative RNA methyltransferase
TEH_22760	*rplV*	3.34	50S ribosomal protein L22
TEH_16440	*dinG*	3.23	Putative ATP-dependent DNA helicase DinG
TEH_00100		3.19	Hypothetical protein
TEH_05920		3.07	Hypothetical protein
TEH_25110		2.95	Hypothetical protein
TEH_11250	*groEL*	2.83	60 kDa chaperonin

existing findings, further efforts are still required to fully decipher the gene components and regulatory mechanisms of salt acclimation networks in *T. halophilus*. Various transcriptome-wide studies suggested that different molecular mechanisms are involved in short-term and long-term salt acclimation. Our study focused on short-term salt acclimation. Therefore, the regulatory mechanism of *T. halophilus* in response to long-term salt stress should be investigated. In addition, gene knockout or gene disruption mutants should be conducted to confirm the functions of candidate genes, and further investigate the regulatory mechanisms of salt tolerance in *T. halophilus* [21, 52]. Moreover, the development of systems biology will contribute to a more comprehensive and thorough understanding by performing an integrated study that couples quantitative iTRAQ-LC–MS/MS proteomics [45], metabolomics [22, 25], lipidomics, and RNA-seq transcriptomics [6, 51].

Acknowledgments Funding for the research presented in this paper was provided by the National Natural Science Foundation of China (Grant 31271924). We also thank RiboBio Co., Ltd (Guangzhou, China) for technical support.

Conflict of interest The authors declare no conflict of interest.

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