Understanding the Mechanism of Thermotolerance Distinct From Heat Shock Response Through Proteomic Analysis of Industrial Strains of Saccharomyces cerevisiae* [S]

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Saccharomyces cerevisiae has been intensively studied in responses to different environmental stresses such as heat shock through global omic analysis. However, the S. cerevisiae industrial strains with superior thermotolerance have not been explored in any proteomic studies for elucidating the tolerance mechanism. Recently a new diploid strain was obtained through evolutionary engineering of a parental industrial strain, and it exhibited even higher resistance to prolonged thermal stress. Herein, we performed iTRAQ-based quantitative proteomic analysis on both the parental and evolved industrial strains to further understand the mechanism of thermostolerant adaptation. Out of ~2600 quantifiable proteins from biological quadruplicates, 193 and 204 proteins were differentially regulated in the parental and evolved strains respectively during heat-stressed growth. The proteomic response of the industrial strains cultivated under prolonged thermal stress turned out to be substantially different from that of the laboratory strain exposed to sudden heat shock. Further analysis of transcription factors underlying the proteomic perturbation also indicated the distinct regulatory mechanism of thermotolerance. Finally, a co-chaperone Mdj1 and a metabolic enzyme Adh1 were selected to investigate their roles in mediating heat-stressed growth and ethanol production of yeasts. Our proteomic characterization of the industrial strain led to comprehensive understanding of the molecular basis of thermotolerance, which would facilitate future improvement in the industrially important trait of S. cerevisiae by rational engineering. Molecular & Cellular Proteomics 14: 10.1074/mcp.M114.045781, 1885–1897, 2015.

Saccharomyces cerevisiae is the most widely used microorganism for large-scale ethanol production in food and beverage industry, and more recently, biofuel industry (1). Yeast industrial strains are renowned for their high ethanol yield and productivity as well as general robustness. However, due to the increasing demand of producing larger and cheaper ethanol volumes worldwide, S. cerevisiae is further challenged with new process requirements. Specifically, yeasts with higher thermostolerance are needed to fulfill fermentation at temperature above 40 °C which will largely reduce cooling costs and help preventing contamination (2, 3). High-temperature cultivation will also benefit a simultaneous saccharification and fermentation process, given that the current compromise between the optimal fermentation temperature (30–35 °C) and saccharification temperature (>50 °C) considerably limits the rate and efficiency of enzymatic hydrolysis (2, 4). Starting from a robust industrial strain of S. cerevisiae, our laboratory has acquired a new diploid strain ScY01 with more superior thermostolerance through the strategy of adaptive evolution. It follows that we hope to identify the molecular basis related to different extent of thermotolerance between the parental and evolved strains. Moreover, it would be equally appealing to distinguish the mechanism of thermotolerance from the classical heat shock response (HSR)1 commonly studied in yeasts.

The laboratory strain of S. cerevisiae S288C has been predominantly used in a number of omic-type studies for characterizing systemic molecular responses of eukaryotic cells to various environmental stresses such as heat shock, ethanol challenge, osmotic stress, and nutrient limitation (5–8). In regard to temperature shock studies, most previous experi-

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* The abbreviations used are: HSR, heat shock response; CCM, central carbon metabolism; TR, thermostolerant response.
Proteomic Analysis of Industrial Yeast Strains

EXPERIMENTAL PROCEDURES

Strains and Yeast Cell Culture—The strains used in this study are listed in supplemental Table S1. Cell culture media and media supplements were purchased from Invitrogen (USA). The parental industrial strain ScY, the evolved strain ScY01 and the laboratory strain S288C were first grown at 30 °C to mid-log phase in YPD medium with 100 g/L glucose. Then cell aliquots of initial OD 0.5 were inoculated into new YPD medium with higher glucose concentration (200 g/L), and cultivated at 40 °C for heat-stressed growth. Controls of the three strains were cultured at 30 °C. After 16 h of growth at normal or elevated temperature, cells of different strains were harvested by centrifugation. Four biological replicates were prepared for each strain grown at a specific temperature.

Protein Extraction, Digestion, and iTRAQ Labeling—Cell pellets of ScY and ScY01 were washed by ddH2O three times before cell lysis. Then cells were resuspended in lysis buffer of 100 mM DTT, 5% SDS, 0.1 m Tris-HCl (pH 7.6) and a complete protease inhibitors mixture (Roche, Mannheim, Germany) before lysed by the glass bead-shaking method. The lysate was centrifuged at 13,000 × g for 10 min to clarify the protein extracts, and supernatants were collected in new tubes. Quantification of the extracted proteins was performed using 2-D Quant Kit (GE Healthcare).

For each sample, 100 μg protein extract was processed and proteolyzed using the FASP protocol with certain modification (19). Specifically, the extract was mixed with 200 μl digestion buffer (8 nM urea in 0.1 m Tris-HCl, pH 8.5), and loaded onto the ultrafiltration cartridge (10 kDa MW cutoff, Millipore, USA). Three washes with the digestion buffer were performed to completely exchange the buffer. Proteins were then reduced with 20 mM DTT for 1 h at 37 °C and alkylated with 40 mM iodoacetamide for 30 min at 25 °C in the dark. Buffer was subsequently replaced with dissolution buffer containing 125 mM TEAB (Sigma) in ddH2O (pH 8.0) by four washes of the ultrafiltration membrane. Protein samples were finally concentrated to ~80 μl in dissolution buffer and transferred to new tubes. Sequencing-grade porcine trypsin (Promega, Fitchburg, WI) was added to each sample with an enzyme to protein ratio of 1:100. Equal amount of trypsin was introduced 2 h later, followed by incubation for another 12 h at 37 °C. Isobaric labeling of individual protein digests from ScY and ScY01 with the 8-plex iTRAQ reagent (Applied Biosystems, Foster City, CA) was conducted according to the user manual. Labeled peptides from 100 μg total proteins of eight different samples were combined prior to LC-MS analysis. Two sample sets were prepared with 8-plex iTRAQ and each one consisted of two biological replicates from ScY or ScY01 grown at 30 °C or 40 °C.

2D-LC-MS/MS Analysis of Labeled Peptide Samples—For each labeled sample set, half of the combined peptides were subjected to 10 RPLC separation. The first dimensional high-pH RPLC was performed on a Nexera UHPLC system (SHIMADZU, Japan) using a 4.6 mm × 250 mm Durashell-C18 column (Agela, China) with a flow rate of 0.8 ml/min. Solvents were composed of water/acetonic acid/formic acid (A: 100%/0%/200 mM, B: 20%/80%/200 mM). The LC method was 0–5 min 5% B, 5–30 min, 5–15% B, 30–45 min 15–38% B, 45–46 min 38–90%, 46–50 min 90%, and re-equilibrium for 10 min at 5% B. The fractionated peptides were collected at one tube/min over the early 45 min and combined to 10 tubes. The resulting 10 fractions were dried in speed vacuum and resuspended in 0.1% formic acid prior to the nanoLC-MS/MS analysis on an Eksigent nanoLC system connected to TripleTof™ 5600 mass spectrometer (Applied Biosystem). After loading on a trapping column, peptides were separated with a gradient of acetonitrile in 0.1% formic acid from 5% to 30% over 70 min at a flow rate of 300 nl/min. MS acquisition settings mainly include ion spray voltage of 2.3 kV, curtain gas of 30 PSI, ion source gas of 4 PSI, and an interface heater temperature of 150 °C. The m/z range for MS and MS/MS scans was

ments utilized microarray approaches to profile changes in gene expression in response to altered cultivation temperatures (9–12). Notably, almost all these transcriptomic analyses focused on abrupt heat or cold shock treatment except one study by Strassburg et al. which characterized the transcriptional and metabolic responses in yeasts adapting to prolonged thermal stress (13). This study concluded that the gradual thermal stress responses of S288C largely agreed with data obtained from the abrupt heat stress induction. Compared with many transcriptomic studies on yeast HSR, only one global proteomic survey has been documented so far (14). The Mann group employed a spike-in stable isotope labeling with amino acids in cell culture approach to measure the changes of the yeast proteome under heat shock stress with nearly complete proteomic coverage (14). The proteomic perturbation reflected strong up-regulation of heat shock proteins and antioxidant proteins as well as suppressed translation-related functions, which are typical characteristics of HSR (15–17).

In contrast to the laboratory strain explored in abundant global analysis of HSR, robust industrial strains have not been subjected to any proteomic surveys for elucidating the mechanism of thermostolerance. Notably, the Nielsen group has very recently conducted genome-wide transcript sequencing of thermostolerant yeast strains evolved from a haploid wild-type strain (18). Their study mainly disclosed specific gene mutations and chromosome segment duplications selected during adaptive evolution upon thermal stress. Interestingly, this elegant work reported a change in sterol composition in the thermostolerant strains caused by both mutation in a single gene and increased expression of several genes involved in sterol biosynthesis (18). As we are aware of the interconnected multi-layer regulation through genomic mutation and transcription/translation, we also conducted genome-sequencing of the evolved and parental strains, and the results will be published elsewhere.

In the present study, we focused on the expression pattern changes in thermostolerant responses of the industrial strain at the proteomic level to infer the transcriptional/translational aspect of the molecular basis. To this end, we performed iTRAQ-based quantitative proteomic analysis on both the parental and evolved industrial strains grown at normal or elevated temperature to clarify the adaptive mechanism. Moreover, the proteomic responses of industrial strains under prolonged thermal stress turned out to be substantially different from that of the laboratory strain exposed to sudden heat shock. Further analysis of transcription factors underlying the proteomic perturbation also indicated the distinct regulatory mechanism of thermostolerance. Finally, two proteins of differential expression were selected to investigate their roles in mediating thermostolerance and ethanol production of yeasts.
set from 350 to 2000 and from 100 to 1500, respectively. Top 30 abundant precursors were selected for MS/MS in each cycle of 3 s. The dynamic exclusion time was set at 25 s. All raw MS data were deposited to a cloud drive (http://pan.baidu.com/s/1jxBe4nn password:uo3w).

Data Analysis for Proteomic Identification and Quantification with iTRAQ Strategy—ProteinPilot™ Software 4.5 (AB SCIEX, Foster City, CA) was used for protein identification. The software performs automatic recalibration such that typical mass errors for MS and MS/MS data were below 10 ppm. The database employed was a Uniprot database for (20). The software performs automatic recalibration such that typical mass errors for MS and MS/MS data were below 10 ppm. The database employed was a Uniprot database for S. cerevisiae (strain S288c, 6621 entries, 07-Jul-2013) supplemented with the trypsin sequence and common protein contaminant sequences. "Thorough ID" searches were performed with trypsin as the specified enzyme (1), 0.05 Da for peptides and MS/MS fragments (20). ProteinPilot automatically clusters the identified proteins into protein groups sharing common peptides. Only protein identifications with >99% confidence were retained, resulting in FDR <1% as calculated by a decoy database search (using a reverse sequence version of the reference database). For quantification analysis, eight-plex iTRAQ was specified to be the quantification method. Only peptides of unique sequences (not shared with other proteins) and free of miscleavages or variable modifications contributed to protein ratio calculation. Protein ratios, p values and error factors (reflecting quantification variation at the peptide level) based on at least two unique peptide ratios were calculated by the software. In experimental sets 1 and 2, only proteins identified with above 99% confidence and quantified with at least two unique peptides are considered quantifiable proteins. Then protein ratios with the error factor below 2.0 (acceptable peptide-level quantification variation) from all biological replicates were retained to derive median ratios. For the statistical test, protein ratios with the error factor below 2.0 and quantified in at least three biological replicates were subjected to ANOVA, and those with p value < 0.001 and a median value > 1.25 or < 0.80 were regarded significantly changed. When analyzing the HSR proteomic data set reported by Mann’s group (14), the aforementioned threshold for the ANOVA test and fold-change were applied to select differential proteins.

Real-time PCR—The ScY01 strain separately grown at 30 °C, 40 °C, and 42 °C to the mid-log phase were harvested by centrifugation at 5000 rpm for 3 min. Total RNA was extracted and purified with RNaseasy mini kit (Qiagen, Valencia, CA) following the user protocol. Reverse transcription was performed using M-MLV Reverse Transcriptase (Promega). Real-time quantitative PCR reactions were performed in 96-Well Optical Reaction Plates (Bio-Rad, Hercules, CA) in duplicates using the FastStart Universal SYBR Green Master (ROX) (Roche), and analyzed on the iCycler IQ5 2.0 Standard Edition Optical System (Bio-Rad). ACT1 gene was used as the internal control. Primers were designed using Primer-BLAST, and they are listed in supplemental Table S2. Biological triplicates were acquired under each growth temperature.

Construction of the MDJ1 and ADH1 Overexpressed Strains—A multicopy plasmid pmCherry(mu)-2µLEU2) constructed in our own lab was used in the overexpression experiment. Mitochondrial DNAJ encoding gene MDJ1 and alcohol dehydrogenase encoding gene ADH1 were amplified from the genomic DNA of ScY01 by PCR. The PCR fragments of MDJ1 and ADH1 genes were separately cleaved and ligated to the plasmid pmCherry(mu)-2µLEU2). The constructed plasmids pmCherry(mu)-2µLEU2)-MDJ1 pmCherry(mu)-2µLEU2)-ADH1 were verified by DNA sequencing. The plasmids were then amplified in E. coli DH5α and isolated with the AxyPrep™ plasmid Miniprep Kit (Axygen, Tewksbury, MA). Transformation of the ScY haploid leucine-auxotroph strain ScYαAuv (supplemental Table S1) with the constructed plasmids or the control plasmid was performed as described (21). The transformed cells were plated on minimal medium, and viable clones were selected and verified using PCR. Yeast plasmids were extracted with the TIANprep Yeast Plasmid DNA Kit (TIANGEN) and used as template in the PCR experiment.

Heat Shock of the ScY Strains with Overexpressed Genes—The MDJ1 and ADH1 overexpressed strains as well as the control strain were cultured at 30 °C for 12 h to enter log phase. Then 1-ml aliquot of each culture was taken and transferred into a new tube which was immersed in 50 °C water bath for 10 min. After heat shock, cells were plated onto regularYPD medium and allowed to grow at 30 °C for 48 h before evaluation of cell viability.

Analysis of Glucose Consumption and Ethanol Yield in Yeasts—To evaluate the ethanol fermentation property of gene deletion strains and overexpression strains used in our study, each engineered strain along with the wild-type strain was cultured in high-glucose YPD medium (200 g/L glucose) at 30 °C or 40 °C, and collected at multiple time points of growth. The collected cells were centrifuged at 10,000 × g for 5 min at 4 °C, supernatants were diluted with ddH2O and filtered through 0.22 μm Nylon Syringe filters prior to HPLC analysis. Glucose and ethanol concentrations were determined using Agilent 1200 HPLC system, equipped with an Aminex HPX-87 i exclusion column (Bio-Rad) maintained at 63 °C. Glucose and ethanol were eluted from the column by 5 mm sulfuric acid solution at a flow rate of 0.6 ml/min and detected with a refractive index (RI) detector. Pure standards (Sigma) were used for identification and quantification of the two compounds from the yeast samples.

RESULTS

Evaluation of thermotolerance of the Parental and Evolved Industrial Strains—In our previous adaptive evolution experiment, we obtained a new diploid strain ScY01 by slow adaptation of the parental industrial strain ScY (a derivative of Ethanol Red, see supplemental Table S1) to increasing cultivation temperature. The evolved strain ScY01 exhibited apparent advantage in growth at 40 °C relative to ScY, and its growth behavior was very similar to that of ScY cultured at normal temperature 30 °C, suggesting better thermostolerant property of ScY01 (Fig. 1 A). In addition, ScY01 was able to consume higher amount of glucose and produce more ethanol than ScY (Fig. 1 B), making it an improved strain of desirable traits for industrial usage. Further comparison with the laboratory strain S288C revealed far better resistance of both ScY and ScY01 strains to prolonged thermal stress in growth (Fig. 1 A). Taking the data point of 16 h cultivation for an example, the growth of S288C at 40 °C at that point was inhibited by 43% relative to growth at 30 °C whereas relative growth inhibition was 16% and only 1% for ScY and ScY01, respectively. In addition, specific growth rate curves of three strains showed ScY and ScY01 grew substantially faster than S288C at 40 °C, again indicating their strong endurance of thermal stress (supplemental Fig. S1 A). All three strains were cultivated with 20% glucose to mimic the fermentation condition for abundant ethanol production.

Quantitative Proteomic Analyses of Industrial Strains in Heat-stressed Growth—To acquire an integrative view of the thermotolerance mechanism of the parental and evolved in-
In industrial strains, we carried out comparative proteomic analysis of ScY and ScY01 strains cultivated at normal versus elevated temperatures using the iTRAQ technique (22). In this experiment, we harvested ScY and ScY01 cells after growth of 16 h at 30 °C (control) and 40 °C (heat-stressed) when different extent of thermotolerance was observed between them and they both displayed supreme endurance to thermal stress compared with the laboratory strain. Total cellular proteins were then extracted, the protein digests were labeled with iTRAQ mass tags for relative protein quantification (Fig. 1C). Each eight-plex iTRAQ experiment analyzed biological duplicates of four different samples, and two individual iTRAQ experiments were performed to acquire four biological replicates for each sample. To avoid possible interference from labeling reagents, the labeling sequence was scrambled in the second experiment. As a result, 3543 unique proteins were identified from ScY or ScY01; among them, 2599 and 2541 proteins had expression ratios quantified in two iTRAQ experiments respectively. The full sets of protein and peptide quantification data are included in supplemental Table S3-S5. The majority of proteins (~60%) were identified with more than 10% of sequence coverage (supplemental Fig. S2A). More importantly, excellent quantification reproducibility was observed between biological replicates (supplemental Fig. S2B). Pair-wise comparison of protein quantification in either strain grown at 40 °C versus 30 °C was concordant among replicates, with Pearson correlation coefficients in the range of 0.77–0.90 for protein ratio measurement for both strains. Proteins quantified in at least three out of the four biological replicates were subjected to the ANOVA-based statistical test, and those with $p$ values $<0.001$ and fold-changes $>1.25$ were considered significantly changed (see the quantification summary in supplemental Table S6). A relatively low threshold was employed here to identify subtle changes that were still supported by the statistical power. Scatter plots revealed that a small subset of proteins showed expression variations in the two strains grown under temperature stress (Fig. 1D), and the number of differential proteins found in ScY01 (204 proteins) were very close to that in ScY (193 proteins). When comparing protein expression levels in ScY01 versus ScY both grown under thermal stress, we observed even less variation in the proteomic profile, with only 5 proteins meeting the criteria for significant changes (Fig. 1D). Therefore, it is speculated that the heat-stressed growth condition (40 °C versus 30 °C) in-

![Fig. 1. Growth evaluation and proteomic analysis of S. cerevisiae strains cultivated at normal or elevated temperature. A, Growth curves of strains ScY, ScY01 and S288C cultivated at 30 °C or 40 °C. They were all grown in high-glucose YPD medium (20% glucose) to mimic the industrial fermentation condition. B, Glucose consumption and ethanol yield measured on ScY and ScY01 grown at 40 °C. C, Schematic of the proteomic experiment on ScY and ScY01 cultured at 30 °C or 40 °C. Cells were harvested at 16 h of growth. Four biological replicates were prepared for each sample in two sets of experiments. D, Proteomic comparison of ScY or ScY01 cultivated at normal or elevated temperature. Protein coverages were plotted against protein ratios measured from cultures at 40 °C versus 30 °C for ScY and ScY01 (left and middle), or from ScY01 versus ScY both cultured at 40 °C (right). Significantly changed proteins are annotated by red spots whose sizes reflect the number of unique peptides used to calculate protein ratios. On top is the histogram of protein ratios for each pair-wise comparison.

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duced larger perturbations in the proteomes of both strains than the intrinsic proteomic differences between them cultivated at the same temperature.

**Comparison of Protein Expression Regulation During Heat-stressed Growth and Heat Shock Response**—An elegant proteomic study by Mann group that investigated the heat shock response of the yeast proteome reported 234 proteins (out of 3152 quantified ones) changing their abundances 30 min after shifting the cultivation temperature from 24 °C to 37 °C (14). To elucidate differences in the molecular basis underlying abrupt heat shock response and prolonged heat-stressed growth, we compared the composition of differentially regulated proteins identified in the previous study and ours. Because the heat shock study defined significant changes of expression solely based on ANOVA analysis, we then applied the 1.25-fold cutoff to yield 155 differential proteins with the expression solely based on ANOVA analysis, we then applied cause the heat shock study defined significant changes of identified proteins in the previous study and ours. Be-

| Functional category                  | ScY-U^a | ScY-D^a | ScY01-U^b | ScY01-D^b | HSR-U^c | HSR-D^c |
|--------------------------------------|---------|---------|-----------|-----------|---------|---------|
| Carbohydrate metabolism              | 5       | 24      | 8         | 18        | 30      | 0       |
| Energy metabolism                    | 0       | 18      | 0         | 17        | 6       | 0       |
| Lipid metabolism                     | 0       | 14      | 0         | 15        | 10      | 1       |
| Amino acid metabolism                | 8       | 32      | 9         | 24        | 8       | 1       |
| Nucleotide metabolism                | 2       | 1       | 3         | 3         | 2       | 2       |
| Cofactors and vitamin metabolism     | 1       | 11      | 2         | 12        | 2       | 0       |
| Glycan biosynthesis and metabolism   | 0       | 4       | 0         | 4         | 0       | 0       |
| Transcription                        | 3       | 3       | 2         | 4         | 2       | 0       |
| Translation                          | 4       | 18      | 4         | 11        | 2       | 12      |
| Folding, sorting and degradation     | 12      | 9       | 20        | 9         | 32      | 1       |
| DNA repair                           | 2       | 1       | 1         | 1         | 0       | 0       |
| Transport & vesicle organization     | 5       | 16      | 7         | 15        | 11      | 1       |
| Signal transduction                  | 2       | 2       | 5         | 4         | 10      | 1       |
| Oxidative stress response            | 2       | 5       | 3         | 5         | 17      | 0       |
| Cell wall biogenesis                 | 0       | 3       | 1         | 4         | 1       | 0       |
| Unknown function                     | 2       | 5       | 4         | 6         | 13      | 0       |
| Total^d                              | 48      | 145     | 67        | 137       | 136     | 19      |

^a The number of up (U)- or down (D)-regulated proteins in ScY cultivated under prolonged thermal stress.
^b The number of up (U)- or down (D)-regulated proteins in ScY01 cultivated under prolonged thermal stress.
^c The number of up (U)- or down (D)-regulated proteins in S288C upon abrupt heat shock from published dataset in ref (14).
^d The total number of non-redundant differential proteins under each condition.

between TR and HSR in the proteomic landscape (Table I and Fig. 2). Growth of the two industrial strains at elevated temperature suppressed expression of a large number of proteins involved in diverse metabolic pathways such as central carbon metabolism, amino acid metabolism, lipid metabolism, cofactor and vitamin metabolism, as well as protein transport and vesicle organization. In contrast, sudden heat shock increased expression of many proteins having functions in carbohydrate metabolism, lipid metabolism, protein folding and degradation, and oxidative stress response (Fig. 2). Interestingly, specific proteins such as cytochrome b2, glycogen phosphorylase, long-chain-fatty-acid-CoA ligase 1, (DL)-glycerol-3-phosphatase, catalase T, and transaminated amino acid decarboxylase were down-regulated in both ScY and ScY01 in TR yet increased their abundances in HSR (supplemental Table S7). It is also noteworthy that in the two processes of TR and HSR, expression of distinct subsets of translation-related proteins were suppressed, suggesting different mechanisms of restricting the translational machinery under two stressed conditions (Fig. 2).

Apart from differentiating proteomic profiles between TR of industrial strains and general HSR of yeast cells, we attempted to distinguish the mechanism of thermostolerance between the parental and evolved strains. Although the proteomic perturbations under thermal stress were highly similar between the two strains, different magnitude or opposite trends of regulation in ScY01 versus ScY was observed on specific proteins of widespread functions in cellular metabolism, transcription and translation, protein folding and sorting, membrane transport, or redox balance (Fig. 2). These distinctive protein expression changes and their connected regulatory network would expand our insight into the effect of
FIG. 2. Heat maps of differentially regulated proteins from ScY or ScY01 strains grown under thermal stress versus normal temperature, or from S288c in heat shock response (HSR). Protein ratios for ScY and ScY01 were medians from at least three biological replicates in our analysis. Protein ratios for HSR were adapted from data sets in ref (14) and processed with the same criteria for significance as ours (see Methods for details). Black squares represent unchanged protein ratios below the significance threshold. Proteins were grouped based on their functional classification in GO and KEGG databases.
adaptive evolution on systemically remodeling the strain’s molecular organization accountable for thermotolerance.

Central carbon metabolism (CCM) has been implicated to play key roles in modulating yeast survival of lethal heat stress, although specific mechanisms of central metabolic genes in regulating thermosensitivity remain unknown (23). A close view of the expression variation of proteins involved in CCM revealed that most glycolytic enzymes increased expression in TR of industrial strains whereas those in the TCA cycle, glycogen and glycerol biosynthesis, and pentose phosphate pathway were largely down-regulated (Fig. 3). Considering key components of the electron transport chain such as

![Diagram of metabolic pathways]

**Fig. 3.** Measured expression changes of enzymes involved in central carbon metabolism from ScY (left square) or ScY01 (right square) strains grown under thermal stress versus normal temperature. Protein ratios were indicated by color code according to the scale. Significantly changed ratios were indicated by black boxes. Proteins without color code did not have quantified data in our study.
cytochrome b/c subunits and ATP synthases also reduced their expression in TR (Fig. 2), we speculated that oxidative phosphorylation was attenuated and glycolysis served as major energy source in two industrial strains grown under thermal stress. Moreover, reinforcing glycolysis as well as undermining other pathways in CCM might be a mechanism exploited by the industrial stains to enrich carbon fluxes for exclusive ethanol fermentation. It is noteworthy that ethanol biosynthesis relying on glycolysis was relatively more active in the evolved strain ScY01 than ScY during heat-stressed growth (Fig. 1B). To clarify the correlation of ethanol biosynthesis with cell growth, we compared specific growth rates (OD/OD/h) of ScY01 and ScY with their specific ethanol production rates (g/L/OD/h) at different time points of culture upon heat stress (SI supplemental Fig. S1). Notably, the specific ethanol production rate reflects the metabolic activity of single cells and is independent of the growth rate of the cell population. The specific ethanol production rate peaked at 30 h of culture while cells reached maximal growth rates much earlier, at 12–16 h of culture (SI supplemental Fig. S1). These data indicate the growth advantage of ScY01 over ScY cannot fully explain its improved ethanol production. Given that up-regulation of the glycolytic pathway was more prominent in ScY01 than ScY in regard to the expression increases of Hxk1, Fba1, Tdh2/3, Pgk1, Gpm1/2, Enol1/2, and Adh1 (Fig. 3), we speculate that modulation of CCM activity in the evolved strain would presumably contribute to higher ethanol yield as well as stronger thermotolerance than the parental strain.

In contrast to selective up-regulation of glycolysis in the industrial strains, general heat shock stress of yeast cells resulted in increased expression of most enzymes in all CCM pathways (Fig. 2). This would provide the stressed cells with maximal amount of ATP and NAD(P)H cofactors required for heat shock protein biosynthesis as well as maintaining cell wall integrity (24–27). Furthermore, the divergent carbon fluxes into multiple pathways could explain the considerably lower ethanol yield in the laboratory strain than the industrial stains.

From the proteins showing different magnitude of expression changes between ScY and ScY01, we selected nineteen of them for RT-PCR analysis to examine transcriptional regulation of the corresponding genes in ScY01 cultivated at 40 °C or 42 °C relative to 30 °C. Eleven out of the 19 genes showed consistent trends of regulation at the transcript-level and the protein-level (supplemental Fig. S3). Furthermore, for the selected genes up-regulated in ScY01 grown at 40 °C versus 30 °C, their expression increase was more significant at 42 °C. Specifically, the mRNA levels of ARG1 and CIT2 in ScY01 at 42 °C was increased by two- to threefold compared with 40 °C (supplemental Fig. S3). The temperature-dependent induction of gene expression may indicate potential roles of these differential genes in protecting industrial strains against long-term thermal stress. We also noticed opposed direction of regulation between transcript and protein levels for the remaining genes in ScY01 (supplemental Fig. S3), which reinforced the necessity of performing proteomic analysis due to the widely appreciated post-transcriptional regulatory mechanism (28–30).

Transcription Factors Associated With Differential Protein Expression in Thermotolerant Response Versus Heat Shock Response—Using YEASTRACT database for regulatory associations of transcription factors (TFs) and target genes in S. cerevisiae, we then searched for TFs of documented associations with proteins found to be differentially regulated in our study. The top-10 TFs ranked by statistical significance of regulating differential genes in HSR and TR are shown in Fig. 4 (see detailed TF-target gene associations in supplemental Table S8). Hsf1 and Msn2/4, the major TFs known to mediate HSR (15), are among the top-10 TFs inferred from the published HSR proteomic data set (14). Seven of the top-10 TFs associated with differential protein expression in therмотolerant responses of ScY and ScY01 overlap, yet a large portion of them are distinct from those related to protein expression changes in HSR (Fig. 4). TFs such as Cst6 and Gcn4 showing specific regulatory connections to TR may reveal new mechan-ism of thermotolerance that is characteristic of the two industrial strains. On the other side, three TFs (Sfp1, Rpn4,
and Hsf1) both associated with HSR and TR could imply similar fundamental pathways underlying the two processes. **Investigation of the Roles of Selected Differential Proteins in Mediating Thermotolerance and Ethanol Biosynthesis of S. cerevisiae**—For functional evaluation of certain differentially regulated proteins, we then tested deletion strains of corresponding genes available in a yeast deletion collection from EUROSCARF (Frankfurt, Germany). Of the nineteen genes subjected to the previous RT-PCR analysis, sixteen have viable deletion strains and we first monitored their growth at normal or elevated temperature. Compared with the wild-type strain S288C, ∆adh1 showed much less growth inhibition at 40 °C versus 30 °C, whereas ∆mdj1 exhibited higher thermosensitivity in heat-stressed growth (Fig. S4A). More dramatic growth difference of ∆adh1 and ∆mdj1 versus the control was observed on solid plates at either normal or elevated temperature (Fig. S4B). ADH1 encodes alcohol dehydrogenase 1, one of the five isozymes catalyzing the interconversion between acetaldehyde and ethanol in yeasts (31, 32). As shown in Fig. S4C, its deletion strain no longer produced ethanol, implying the dominant role of ADH1 in ethanol biosynthesis. The evident growth advantage of ∆adh1 could be mainly attributed to abolished synthesis of ethanol which is known to suppress cell growth and glucose uptake (33–37). However, glucose consumption was also reduced in ∆adh1, suggesting possibly lowered activity of glycolysis (Fig. S4C). Interestingly, this strain exhibited lower growth inhibition at elevated temperature than the wild-type (Fig. S4A and S4B), implying stronger resistance to thermal stress. This finding prompted us to speculate other metabolic pathways might be modulated in ∆adh1 to generate heat-resistant metabolites such as glycerol or fatty acids when ethanol production was halted (38–40). As for MDJ1 encoding a member of the Hsp70 chaperone family, its depletion resulted in more profound growth inhibition at 40 °C versus 30 °C. Moreover, ethanol biosynthesis in ∆mdj1 was completely shut down when grown at 40 °C though its ethanol yield was close to the level of wild-type at 30 °C. Therefore, MDJ1 was specifically required for the growth and ethanol production of yeast cells under thermal stress.

We further assessed the function of ADH1 and MDJ1 by overexpressing them in the industrial strain ScY. The MDJ1 overexpressed strain restored better growth than wild-type ScY after abrupt heat shock treatment (Fig. S5A). When exposed to prolonged thermal stress, the MDJ1 overexpressed strain grew a little better than the wild-type in the lag phase and log phase, and also yielded more ethanol. But cell growth and ethanol biosynthesis became close to the wild-type when the overexpressed strain entered the stationary phase (Fig. S5B). These results implicated MDJ1 plays certain roles in protecting cells against heat stress in the exponential growth period, and long-term tolerance to the stress might need a complex chaperon network coordinating functions of multiple chaperon proteins. The ADH1 overexpressed strain did not show alteration in growth or ethanol biosynthesis (Fig. S5B).

**DISCUSSION**

Enduring high-temperature stress over the fermentation process is a highly valuable trait of yeast industrial strains that can largely reduce the production expense (2, 3). The present study acquired an integrated view of the thermotolerant response from two industrial yeast strains at the proteomic landscape. This proteomic footprint of the thermotolerant response was found to be drastically different from that of the heat shock response of yeasts. Our study has strengthened the importance in distinguishing a tolerance response from a stress response, as the commonly observed stress response is not always a tolerance characteristic and does not necessarily lead to a tolerance withstanding the environmental stress. Only through elucidating the thermotolerance mechanism can we possibly identify active functional characteristics that are accountable for the strain adaptation to prolonged thermal stress.

**Differential Proteomic Patterns of Industrial Strains in TR Versus General HSR**—Not surprisingly, when the industrial strains were cultivated at elevated temperature to induce a thermotolerant response (TR), their proteomic profiles were perturbed in a variety of biological processes (Fig. 5) with the HSP family among the most profoundly regulated functional groups. It is well known that the classical responses to heat shock involve the induction of HSPs including chaperones and proteases that are required for protein refolding and degradation (9, 41–44). Notably, fewer HSPs underwent up-regulation in TR than in conventional HSR (Fig. 5). For instance, Ssa4 exhibiting nascent polypeptide refolding activity (45, 46) and Hsp42 required to maintain the solubility of various yeast proteins during heat shock (47, 48) were significantly up-regulated in the previous proteomic and transcriptomic analysis of HSR (15, 43), but they remained unchanged under the thermotolerant condition in our study. Conversely, Mdj1, a HSP cochaperone, increased its expression by 1.5 fold in the ScY01 strain grown under thermal stress whereas showed no expression change in HSR. Mdj1 is reported to facilitate the activation of Ssc1, the crucial Hsp70 chaperone responsible for protecting nascent polypeptides and refolding damaged proteins in mitochondria (49, 50). Because our proteomic analysis also revealed the abundance of Ssc1 was not significantly changed in TR, we speculated Ssc1 activity may be enhanced mainly through up-regulation of Mdj1. Our further investigation of Mdj1 function demonstrated this cochaperone was required for yeast growth at elevated temperature, and its overexpression in the parental industrial strain could improve cell viability and ethanol production to some extent in the actively growing phase under thermal stress (Fig. S4 and S5). As for proteasome subunits and proteases commonly induced in HSR for eliminating misfolded and damaged proteins (44), they were unchanged or even down-regulated in
TR, which indicated the tolerance mechanism no longer involves activation of the protein degradation machinery. These findings collectively implied the industrial strains rely on regulation of a minimal pool of HSPs which are likely to be essential for maintaining thermotolerance under prolonged stress. This would allow cells to lower the energy burden of synthesizing as many new HSPs as in HSR.

Another distinct feature of thermotolerant industrial strains is that they repressed the expression of various metabolic enzymes involved in TCA cycle, pentose phosphate pathway, starch/sucrose metabolism, fatty acid metabolism, and amino acid metabolism (Fig. 5). In contrast, the majority of these enzymes substantially increased their expression during HSR, to provide building blocks for synthesis of HSPs as well as lipids and carbohydrate needed for cell membrane integrity (26, 51, 52). Concordantly, in HSR, all central metabolic pathways as well as oxidative phosphorylation were enhanced with more enzyme availability for adequate supply of ATP and NAD(P)H required for biomolecule synthesis. However, the industrial strains seem to considerably diminish their energy demand in TR, and the remaining up-regulated glycolytic pathway is assumed to restrict carbon fluxes and energy exclusively to ethanol fermentation. Notably, central metabolic enzymes such as Pfk2 (phosphofructokinase) and Cit2 (citrate synthase) were previously reported to be required for yeast survival of lethal heat shock (23). Thus, their modest up-regulation observed in our study might promote growth of industrial strains under prolonged thermal stress. In addition, deletion of another glycolic enzyme Adh1 (alcohol dehydrogenase) was shown in our study to not only abolish ethanol biosynthesis but enable less growth inhibition at elevated temperature. The precise roles of these central carbon metabolic enzymes in modulating thermosensitivity are largely unknown and will be the subject of future studies.

Although most enzymes participating in amino acid metabolism were down-regulated in TR, a small subset including Arg1, Arg 3, Arg 5/6, Leu 2, and His5 showed strong up-regulation (Fig. 5, supplemental Table S7). The first three play roles in both arginine and proline biosynthesis. It is of particular notion that proline is recognized as a protectant against oxidative, ethanol, and osmotic stress in yeast, yet proline metabolic enzymes did not vary expression levels in the laboratory strain under general environmental stresses (53). Therefore, specific regulation of Arg enzymes in TR suggested the industrial strains may synthesize more proline to enhance their stress resistance. In accordance with this speculation, the proline degradation enzyme Put1 reduced expression by 40% in the industrial strains grown under thermal stress. Previous studies have shown that yeast strains with disrupted PUT1 gene acquired stronger tolerance to diverse environmental stresses (53). Taken together, the industrial yeast strains may have evolved a new mechanism of enhancing its general stress tolerance by mediating proline biosynthesis and catabolism.

Oxidative stress is presumably induced after the primary stress of heat shock as a function of oxygen availability (15). Therefore, in classical HSR, a panel of antioxidant proteins such as Ctt1 (catalase), Grx1/Grx2 (glutaredoxins), Tsa1 (peroxiredoxin), Sod1/Sod2 (superoxide dismutases) and Trx1 (thioredoxin) all significantly increased expression to counteract the heat-induced oxidative stress (5, 54). However, the industrial strains seemed to experience much less severe oxidative stress in TR as only a handful of oxidoreductases (e.g. Grx1, Sod1) were up-regulated during heat-stressed
growth (Fig. 5). Moreover, Ctt1 and Trx1 were even down-regulated under the thermotolerant condition. Thus, it is inferred that long-term adaptation to temperature stress could alleviate the accompanying oxidative stress, again reducing the energy cost needed for oxidative defense.

**Differential Proteomic Patterns of the Parental Strain Versus the Evolved Strain in TR**—In addition to revealing distinct proteomic responses related to thermotolerance in contrast to general heat shock, our study also shed light on differentiating the tolerance mechanism between the parental industrial strain (ScY) and the evolved strain (ScY01). Subsets of proteins in diverse functional categories were differentially expressed in ScY01 yet unchanged in ScY during the thermotolerant response (Fig. 2). Particularly interesting are specific HSPs (Ssa1, Sse1/2, Hsp104, Aha1, and Mj1), signaling factors (Sgt1, Rdi1, and Glc7), and proteins involved in vacuole transport or targeting (Pep4, Pep1, Mog1, Arp1, and Yip3). Notably, Hsp104 is one of the few yeast chaperones absolutely required for survival of lethal heat shock and strongly induced in HSR (15, 43, 55, 56), and it was also up-regulated in ScY01 grown under thermal stress. Sse1/2 proteins are cochaperones of Hsp70 which plays major roles in up-regulated in ScY01 grown under thermal stress. Sse1/2 target genes such as HSPs (60). Msn2/4 activation is known to be prominent in the evolved strain than in the parental strain during TR (Fig. 4). Snf2 is the catalytic subunit of the Swi/Snf chromatin remodeling complex (65). Transcriptomic analysis of Snf2 defective cells has revealed its necessary roles in the activation and repression of distinct gene sets especially those involved in ribosomal biogenesis upon heat shock (66). In summary, our analysis of HSR- and TR-responsive TF sets suggested that different combination of a fairly small number of responsive TFs may be the molecular basis for distinguishing the two processes.

**Differential Transcription Factor Associations With TR versus HSR Inferred From Global Proteomic Variation**—The wide array of differentially regulated proteins associated with TR or HSR allowed us to infer potential coregulation of multiple TFs under a specific condition (Fig. 4). As expected, Hsf1, Msn2/4, and Yap1 were among the top-10 TFs showing most significant associations with differential gene regulation in HSR. Hsf1 is the primary TF dedicated to regulate HSR by recognizing a heat shock element located in the promoters of its target genes such as HSPs (60). Msn2/4 activation is known to trigger a so-called environmental stress response so as to adapt yeast cells to various adverse conditions including ethanol, heat shock, chemical toxic, osmotic stress, and oxidative stress (15, 61). Yap1 mainly regulating oxidative stress response and redox homeostasis was also expected to be the major player in HSR due to the well-recognized intersection between heat shock stress and oxidant defense (62). Disappearance of Msn4 and Yap1 from the top 10 TF list for TR suggested thermotolerant strains may rely more on heat stress-specific regulatory mechanism rather than initiating general stress responses or oxidative defense.

Both Cst6 and Gcn4 are significantly associated with more than 60% of differential protein expression in industrial strains grown under heat stress (Fig. 4). Interestingly, Cst6 belongs to five novel heat-responsive TFs recently identified using a gene regulatory module which was constructed based on TF-promoter binding matrix and heat shock gene expression data (63). In addition, Cst6 is reported to regulate genes involved in the cell cycle and DNA processing (63). Therefore, much more significant correlation of Cst6 to the proteomic variation observed in TR than in HSR implied a potential crosstalk between cell cycle modulation and thermotolerant adaptation. Gcn4 is a master activator of amino acid biosynthetic genes under diverse stress conditions (64). Its prominent association with protein expression in TR rather than HSR indicated that the industrial stains may experience amino acid starvation in heat-stressed growth as a result of down-regulation of many amino acid metabolic enzymes. Furthermore, Snf2 is a TF found in our study to more significantly regulate protein expression in the evolved strain than in the parental strain during TR (Fig. 4). Snf2 is the catalytic subunit of the Swi/Snf chromatin remodeling complex (65). Transcriptomic analysis of Snf2 defective cells has revealed its necessary roles in the activation and repression of distinct gene sets especially those involved in ribosomal biogenesis upon heat shock (66).

In summary, our analysis of HSR- and TR-responsive TF sets suggested that different combination of a fairly small number of responsive TFs may be the molecular basis for distinguishing the two processes.

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This article contains supplemental Figs. S1 to S5 and Tables S1 to S8.

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