Saccharomyces cerevisiae Cytosolic Thioredoxins Control Glycolysis, Lipid Metabolism, and Protein Biosynthesis under Wine-Making Conditions

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ABSTRACT Thioredoxins are small proteins that regulate the cellular redox state, prevent oxidative damage, and play an active role in cell repair. Oxidative stress has proven to be of much relevance in biotechnological processes when the metabolism of *Saccharomyces cerevisiae* is mainly respiratory. During wine yeast starter production, active dry yeast cytosolic thioredoxin Trx2p is a key player in protecting metabolic enzymes from being oxidized by carbonylation. Less is known about the role of redox control during grape juice fermentation. A mutant strain that lacked both cytosolic thioredoxins, Trx1p and Trx2p, was tested for grape juice fermentation. Its growth and sugar consumption were greatly impaired, which indicates the system's relevance under fermentative conditions. A proteomic analysis indicated that deletion of the genes *TRX1* and *TRX2* caused a reduction in the ribosomal proteins and factors involved in translation elongation in addition to enzymes for glycolysis and amino acid biosynthesis. A metabolomic analysis of the *trx1Δ trx2Δ* mutant showed an increase in most proteogenic amino acids, phospholipids, and sphingolipids and higher fatty acid desaturase Ole1p content. Low glycolytic activity was behind the reduced growth and fermentative capacity of the thioredoxin deletion strain. All three hexokinases were downregulated in the mutant strain, but total hexokinase activity remained, probably due to posttranslational regulation. Pyruvate kinase Cdc19p presented an early level of aggregation in the *trx1Δ trx2Δ* mutant, which may contribute to a diminished hexose metabolism and trigger regulatory mechanisms that could influence the level of glycolytic enzymes.

IMPORTANCE Oxidative stress is a common hazardous condition that cells have to face in their lifetime. Oxidative damage may diminish cell vitality and viability by reducing metabolism and eventually leading to aging and ultimate death. Wine yeast *Saccharomyces cerevisiae* also faces oxidative attack during its biotechnological uses. One of the main yeast antioxidant systems involves two small proteins called thioredoxins. When these two proteins are removed, wine yeast shows diminished growth, protein synthesis, and sugar metabolism under wine-making conditions, and amino acid and lipid metabolism are also affected. Altogether, our results indicate that proper redox regulation is a key factor for metabolic adaptations during grape juice fermentation.

KEYWORDS *Saccharomyces cerevisiae*, fermentation, glycolysis, metabolomics, oxidative stress, proteomics, thioredoxin-thioredoxin reductase systems, wine, yeasts

The yeast *Saccharomyces cerevisiae* adapts to adverse situations in nature through complex stress response mechanisms that protect it against cellular damage (1). The same applies to the harmful conditions that yeast cells face in every step of their

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biotechnological use, from their propagation as starters to be used in food production to their role during industrial fermentations to produce food, energy, or pharmaceuticals (2). Many stressful environmental and metabolism-derived conditions have an impact on yeast performance during grape juice fermentation to produce wine (3). One of these is oxidative stress. Eukaryotic organisms have an oxygen-dependent metabolism because oxygen is selected as the final electron acceptor in the respiration process. This is why these organisms are submitted to the oxygen paradox (4). Cells/organisms depend on oxygen for their survival during respiratory metabolism. At the same time, however, their oxidant power causes cytotoxic compounds called reactive oxygen species (ROS). The most powerful intracellular source of ROS is oxidative phosphorylation through the electron transport chain in mitochondria (5). These events are not limited to only the typical respiratory conditions of biomass production (6). Fermenting wine yeasts accumulate ROS, particularly at the end of fermentation (7, 8), and they suffer oxidative damage in molecular terms (7). Antioxidant proteins also contribute to longevity under enological conditions (8). The antioxidant response under this condition is poorly defined and may be relevant for industrial yeast performance.

S. cerevisiae has many mechanisms against oxidative stress; some detoxify cellular ROS, others prevent cellular damage, and others repair this damage (9). Thioredoxins are small oxidoreductases that are involved in protection and reparation mechanisms (10). The cytoplasmic thioredoxin (cTRX) system is formed by two thioredoxins, Trx1p and Trx2p, and a thioredoxin reductase, Trr1p, that provides reducing power. A mutant strain for both thioredoxins is auxotrophic for sulfur amino acids because thioredoxins are the hydrogen donor to 3'-phosphoadenosine-5'-phosphosulfate (PAPS) reductase, which converts PAPS into sulfite (11). Cytoplasmic thioredoxin deletion also causes a defect in the S phase of the cellular cycle because thioredoxins are responsible for ribonucleotide reductase (RNR) reduction to keep RNR active (12). In ROS detoxification terms, thioredoxins confer reducing power to thioredoxin peroxidases or peroxiredoxins, which eliminate H₂O₂ and other peroxides. Previous studies from our laboratory have shown the importance of the thioredoxin system for protecting against oxidative stress during the industrial biomass propagation process. A strain overexpressing the *TRX2* gene produced higher biomass yield during growth on molasses (13). Moreover, the aforementioned biomass presents increased fermentative capacity and a shorter lag phase as a result of increased defense against oxidative stress, which diminished lipid and protein oxidation. Our laboratory studied the role of Trx2p in glucose metabolism during biomass propagation at the hexokinase level. *TRX2* overexpression increased not only the *HXK2* mRNA levels but also the amount of the Hxk2p protein and enzyme activity (14). *TRX2* overexpression also protected other glycolytic enzymes against oxidation during the biomass propagation process (15).

Here, we studied the role of the cytosolic thioredoxin system in grape juice fermentation to evaluate the relevance of redox-controlling systems under wine-making conditions, where the relevance of these systems is less well characterized. We show that the *trx1Δ trx2Δ* double mutant displays slower growth for this condition. By using global proteomic and metabolomics assays, we found a strong impact on metabolism that involved alterations in the levels of key metabolic proteins, such as hexokinases and fatty acid dehydrogenase. We found that some pathways, like glycolysis and fatty acid biosynthesis, are influenced by the cTRX system. With glycolysis, this could be related to the aggregation of some enzymes like pyruvate kinase Cdc19p.

RESULTS

The cytosolic thioredoxin system plays an important role in growth during grape juice fermentation. In order to study the impact of the Trx1p and Trx2p system on industrial yeast and its implication for wine-making conditions, the deletion mutants of paralog genes *TRX1* and *TRX2*, as well as the *trx1Δ trx2Δ* double mutant, were built in a haploid wine industrial yeast known as C9 (16). This haploid strain was chosen to simplify the genetic manipulation process, as previous results have shown its proper performance for wine making (17). To study the role of cytosolic thioredoxins, the

FIG 1 *TRX1* and *TRX2* deletion impacts growth and longevity in grape juice. (A) Viable cell number in the synthetic grape juice MS300 fermentations of the C9 wine strain and *trx1Δ*, *trx2Δ*, and *trx1Δ trx2Δ* deletion mutants. (B) Survival curve during fermentations. The viable cell number on day 3 from panel A was taken as 100% survival. (C) Sugar consumption during the above-described fermentations. (D) Ethanol production at the end of fermentation. Experiments were done in triplicate. Means and standard deviations are provided. *, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.005$, unpaired t test, two-tailed.

mutants were tested in synthetic grape juice MS300 (Fig. 1), a standard medium which allowed us to perform global analyses that can be compared with previous works conducted with the same growth medium (18–20). Growth was followed by counting viable cells (Fig. 1A). No significant differences were observed in the growth between the wild-type and simple mutants, but both had a slightly lower maximal cell density. This indicates that these two homologous genes are functionally redundant under fermentative conditions. However, the *trx1Δ trx2Δ* double mutant had a significantly slower growth rate and a lower maximal cell density but higher viabilities for longer times (Fig. 1A). The viability count allowed us to measure chronological life span (CLS) by taking the wild type's (wt) highest point of cell viability (shown in Fig. 1A) as 100% survival. This allowed us to obtain a CLS plot under wine-making conditions (Fig. 1B). In this case, the survival of *trx1Δ* and *trx2Δ* strains increased slightly before dropping similarly to that of the wild-type strain, while the *trx1Δ trx2Δ* mutant dramatically extended the CLS and was the only strain with over 10% viability after 2 weeks. Sugar consumption during fermentation is shown in Fig. 1C. Although the *trx2Δ* mutant was slightly delayed, both single mutants finished sugars at the same time as the wild type, which reinforces the idea that the two proteins play redundant roles. The *trx1Δ trx2Δ* double mutant displayed a slowed sugar metabolism, which once again indicated the relevance of the cTRX system under fermentative conditions. The *trx1Δ trx2Δ* strain was able to eventually end fermentation and then produce a similar ethanol amount to the reference strain and the simple mutants (Fig. 1D). Thus, sugar metabolism was slowed down but not impaired. Similar results were obtained when these mutants were tested

FIG 2 *TRX1* and *TRX2* deletion impacts specific protein abundance in must fermentations. (A) Functional analysis of the proteins enriched in the wild-type strain in relation to the *trx1Δ trx2Δ* double mutant. (B) Cycloheximide sensitivity assay. The inhibition halo caused by 1 mM cycloheximide is shown. Experiments were done in triplicate. Means and standard deviations are provided. (C) The interactome node based on the proteins enriched in the *trx1Δ trx2Δ* mutant according to String (21). Colors are random.

in natural grape juice (see Fig. S1 in the supplemental material), thus supporting the relevance of the cytosolic thioredoxin system under fermentative conditions. As *TRX2* gene overexpression improves biomass production in wine strain T73 (13), a *ura3* version of strain C9 was transformed with a multicopy plasmid containing the *TRX2* gene. *TRX2* overexpression did not impact either cellular growth or sugar consumption during synthetic grape juice MS300 fermentation in either genetic background (see Fig. S2 in the supplemental material). This result indicates that thioredoxin is not limiting during wine making.

***TRX1* and *TRX2* deletion alters the global proteome during fermentation.** To gain a better understanding of the role of the cytosolic thioredoxin system in grape juice fermentation, a quantitative study of the differential proteomes of wild-type strain C9 and the *trx1Δ trx2Δ* double mutant was performed. Samples were taken in the stationary phase in synthetic grape juice MS300 fermentations when 50 g/liter of hexoses still remained. The stationary phase is a steady state regarding cell cycle, so yeasts do not divide and different strains can be compared without the interference of growth while they are actively fermenting as plenty of sugar remains. Only one time point was taken due to technical limitations, but events at the very beginning of growth could influence all fermentation (see below). Of the 502 detected proteins, 224 displayed statistically significant abundance changes (a fold change above 1.5) between the *trx1Δ trx2Δ* double mutant and the wild-type strain (see Table S1 in the supplemental material). The String program (21) was used to detect enrichment in the functional categories in the 203 more abundant proteins in the wild-type strain than in the *trx1Δ trx2Δ* double mutant (Fig. 2A). According to biological processes, this group

of proteins is enriched in components of both the “cytosolic translation” category, particularly in the elongation step, and the “metabolic processes” category, such as monosaccharide metabolism. Regarding the enriched KEGG pathways in this set of proteins, once again translation (in the form of ribosome) was the most significant pathway, together with metabolic pathways, including both amino acid biosynthesis and glycolysis/gluconeogenesis (and, therefore, hexose metabolism once again). Aminoacyl tRNA synthetases were less abundant in the mutant, which could impact translation elongation. To confirm the impact of the mutation on translation, sensitivity to cycloheximide (a drug that targets translation elongation) was tested (Fig. 2B). The mutant strain displayed increased sensitivity, which confirmed the translation defect. Diminished translation machinery could explain the lower growth rate of the thioredoxin mutant, together with decreased amino acid metabolism. One of the most important protein families detected in the proteome analysis that could influence sugar metabolism and growth (see below) belonged to hexokinases. According to the transcription factor YeastRACT database (22), 93.56% of the genes that code for the proteins detected by proteome analysis are regulated by a well-known regulator of ribosomal genes known as Sfp1p. Interestingly, 88.12% of genes are regulated by Yap1p, the main oxidative stress-responsive transcription factor, which could be a way to alter protein levels in the thioredoxin mutant.

There were only 21 proteins that were statistically significantly more abundant in the *trx1Δ trx2Δ* double mutant than in the wild-type strain. With the String program, no enrichment was observed in any functional category, but several proteins showed a relationship based on interaction, which suggests a common function in mitochondrial energy metabolism (Fig. 2C). Cor1p, Cox2p, Por1, Mcr1, Mir1p, Ole1p, and Atp1p displayed a relationship by establishing a node, and mitochondrial phosphate carrier Mir1p was the protein with the highest fold change of the whole proteome. According to YeastRACT, they are all regulated by general stress transcription factor Msn2p. Actually, the product of a well-known Msn2/4p target gene, Hsp12p, was increased by 5.8-fold in the mutant. The presence of the only fatty acid desaturase, Ole1p, in this group of proteins is noteworthy, and its relevance is discussed below. As a compensatory mechanism, thioredoxin reductase Trr1p increased in the mutant but with oxidative stress proteins being scarcely induced, except for NADPH oxidoreductase Oye3p (2.0-fold) and glutathione (GSH) S-transferase omega like-2 Ecm4p (14.0-fold). The striking upregulation of the latter could be responsible for the marked decrease in GSH (see below). Therefore, the proteomic analysis did not indicate an oxidative stress response under wine-making conditions due to the absence of thioredoxin and according to the parameters expected in aerobic growth, although mechanisms such as the glutathione system (see below) could compensate for this loss. This indicated a mainly redox regulatory function for Trx1p and Trx2p under wine-making conditions, but the importance of their antioxidant function cannot be ruled out.

Lack of thioredoxins alters the global fermentative metabolome. The metabolomes in the *trx1Δ trx2Δ* mutant and its parental strain were analyzed under the same conditions as the proteomic analysis, i.e., in actively fermenting stationary phase in synthetic grape juice MS300 (see Table S2 in the supplemental material). The principal-component analysis (PCA) of the five samples per strain showed that the deletion of *TRX1* and *TRX2* strongly impacted the global metabolism compared to that of the parental strain (Fig. 3A). This analysis detected 423 metabolites in all, and 264 showed differences between strains with a significant *P* value (≤ 0.05). Of these, 197 were more abundant in the *trx1Δ trx2Δ* mutant, and only 67 metabolites were less abundant in the *trx1Δ trx2Δ* strain (Table S2). All of the proteinogenic amino acids, except proline, cysteine, glycine, and arginine, were higher in the *trx1Δ trx2Δ* double mutant than they were in the wild type. Therefore, the relatively smaller amount of amino acid biosynthetic proteins in the mutant strain could reflect a feedback repression mechanism. The modified amino acids, such as acetylated amino acids, were also higher in the *trx1Δ trx2Δ* double mutant. As these amino acid modifications occurred posttranslationally,

FIG 3 *TRX1* and *TRX2* deletion impacts global metabolism under wine-making conditions. (A) PCA of the metabolomic data from the wild type and the *trx1Δ trx2Δ* double mutant. (B) Diagrams showing total and oxidized glutathione and ergosterol in both C9 and the *trx1Δ trx2Δ* double mutant. (C) Effects on the fatty acid biosynthetic pathway. The upregulated proteins and metabolites in the *trx1Δ trx2Δ* strain are highlighted in red and the downregulated ones in green. Five replicates of each strain were tested in the metabolome analysis, and three replicates were used for the proteome analysis.

their presence implied proteolysis, suggesting that the higher levels of amino acids observed in the *trx1Δ trx2Δ* double mutant could be due to a larger amount of proteolysis in these cells than in the wild-type strain. This could be caused by the mutant's inability to repair oxidized proteins or to prevent their damage. Many amino acid catabolites, such as kynurenine, kynurenate, 4-hydroxyphenylpyruvate, saccharopine, and pipicolate, were also higher in the *trx1Δ trx2Δ* double mutant than in the wild-type strain. However, it was noteworthy that the lysine catabolites 2-amino adipate and glutarate, downstream of saccharopine, were, respectively, 10- and 5-fold lower in the *trx1Δ trx2Δ* double mutant than in the reference strain. Coherently, the enzyme that catalyzes the conversion of saccharopine into 2-amino adipate, saccharopine dehydrogenase, was 6-fold downregulated in the *trx1Δ trx2Δ* double mutant (see Table S1). A 10-fold accumulation of 4-imidazoleacetate, a catabolite of histidine, and a 5-fold accumulation of isocitrate compared to levels in the wild type were also observed in this mutant. All of these compounds were substrates for a dehydrogenase reaction in which NAD⁺ or NADP⁺ are reduced to NADH or NADPH. We found no reports on the direct involvement of thioredoxin in the functioning of these dehydrogenase enzymes. The altered overall redox state of the *trx1Δ trx2Δ* double mutant could have an effect

on these reducing enzymatic reactions. The larger content of the oxidized glutathione (GSSG) and the approximately 3-fold lower content of the reduced glutathione (GSH) in the mutant (Fig. 3B) could further substantiate this premise. High levels of γ -glutamyl amino acids and a trend of higher 5-oxoproline suggested that the glutathione cycle was induced in the *trx1Δ trx2Δ* double mutant, possibly as a mechanism to compensate for the loss of the thioredoxin system. The abundance of cystine and hydroxyl fatty acids reinforced the idea of an oxidative environment caused by cTRX deletion.

Some pathways were affected by the deletion of *TRX1* and *TRX2* in both proteomic and metabolomic terms. One pathway with major differences in the abundance of proteins and metabolites was fatty acid biosynthesis (Fig. 3C). Ole1p, $\Delta(9)$ fatty acid desaturase, is the only fatty acid desaturase described in *S. cerevisiae*. In the proteome analysis, Ole1p was detected as one of the most abundant proteins in the *trx1Δ trx2Δ* mutant (4-fold more in the *trx1Δ trx2Δ* strain than in the parental strain) (see Table S1). This could explain the increase in unsaturated fatty acids, such as oleate and palmitoleate. Two other altered proteins in the *trx1Δ trx2Δ* strain were fatty acid synthetases Fas1p and Fas2p, which were less abundant in the *trx1Δ trx2Δ* mutant than in the wild type. Short fatty acids, such as caprate and laureate, decreased accordingly. Despite the reduction in Fas1/2p in protein terms, the final levels of long fatty acids like stearate and arachidate in the double mutant were high as were the levels of phospholipids, sphingolipids, and conjugated fatty acids, suggesting regulation in enzymatic activity or lower lipid degradation in the mutant. Lysophospholipase 1 Plb1 was greatly repressed (10.8-fold) in the mutant strain (Table S1). Sterols increased in the thioredoxin mutant metabolome, particularly ergosterol, which was the final product of this pathway (Fig. 3B), with no change noted in the levels of the enzymes involved in its biosynthesis. This suggested a regulation in enzymatic activity or better ergosterol uptake from the medium, which could compensate for the oxidative damage caused in membrane fatty acids. All together, these data indicate changes in membrane composition that could influence the increased survival of the *trx1Δ trx2Δ* double mutant (Fig. 1B).

Cytosolic thioredoxins influence hexose metabolism. As the proteins involved in carbon metabolism in general, and in glycolysis in particular, were downregulated in the thioredoxin mutant, we focused on this central metabolic pathway (Fig. 4). The downregulated proteins in the *trx1Δ trx2Δ* double mutant (*trx1Δ trx2Δ*/wt strain ratio, ≤ 0.66) are highlighted in green. None of the enzymes of the pathway increased due to the mutation, but several enzymes were less abundant in the *trx1Δ trx2Δ* strain than in the wild type. The most striking difference affected the first step during glycolysis and involved hexose phosphorylation, as the *trx1Δ trx2Δ* double mutant had lower levels of the three proteins for hexose phosphorylation: hexokinase 1 (Hxk1p), hexokinase 2 (Hxk2p), and glucokinase 1 (Glk1p) (see Table S1 in the supplemental material). This would explain the lower glucose consumption rate by the mutant than by the parental strain (Fig. 1C). In parallel, the metabolites produced by these enzymes were also affected, and lower levels of glucose-6-phosphate and glucose-1-phosphate were detected in the *trx1Δ trx2Δ* strain, which could contribute to its growth defect during grape juice fermentation. Pfk2p, one of the subunits of phosphofructokinase that catalyzes the third glycolytic step, was downregulated (2.5-fold) in the mutant, but its product, fructose 1,6-bisphosphate, was the only glycolytic metabolite to significantly overaccumulate in the mutant strain. The even bigger decrease (3.9-fold) in the level of the enzyme catalyzing the fourth step in the pathway, aldolase Fba1p, could be responsible for this fructose 1,6-bisphosphate accumulation, but it could also reflect the slowdown of the nonoxidative branch of the pentose phosphate pathway. Additional enzymes in the lower part of the pathway, e.g., Tdh3p, Eno2p, Gpm1p, and Pgc1p, also decreased in the wild type. However, no significant change was noted in the amount of metabolites in the lower part of the pathway, including pyruvate, despite the lower levels (5.6-fold) of the main pyruvate kinase Cdc19p. Minor pyruvate decarboxylase Pdc6p was 4-fold more abundant in the *trx1Δ trx2Δ* strain than in the parental strain,

FIG 4 Double thioredoxin mutant has a defect in hexose assimilation. The glycolytic proteins and metabolites upregulated in the *trx1Δ trx2Δ* double mutant are depicted in red and the downregulated (Continued on next page)

FIG 5 Thioredoxin mutation impairs growth and fermentative capacity. (A) The C9 and *trx1Δ trx2Δ* double mutant growth curves in YPD (2% glucose) measured by an optical density plate reader. (B) As for panel A, but in YPF (2% fructose). (C) As for panel A, but in synthetic must MS300. (D) Fermentative capacity measured as CO₂ production during 6 h of growth in synthetic grape juice. Data correspond to representative data from three replicate experiments.

while Pdc1p, the main pyruvate decarboxylase, decreased by 3-fold (Table S1). Either these changes could cancel one another out or additional processes took place for pyruvate to remain unchanged, and this could be the reason underlying the unchanged final amount of ethanol after fermentation (Fig. 1D).

Thioredoxin influences growth, fermentative capacity, hexokinase activity, and pyruvate kinase aggregation. To check whether the alteration in glycolysis in the thioredoxin mutant impacted yeast physiology under different conditions, growth was measured as the increase in optical density at 600 nm (OD₆₀₀) in laboratory media containing different sugars as carbon sources: a rich medium with 2% glucose (yeast extract-peptone-dextrose [YPD]), a rich medium with 2% fructose (yeast extract-peptone-fructose [YPF]), and synthetic grape juice MS300 (with 200 g/liter of sugars, both glucose and fructose) used as the control (Fig. 5A, B, and C). In the YPD and YPF media (Fig. 5A and B), an extended lag phase was noted in the *trx1Δ trx2Δ* double mutant strain, and the final cellular density was lower than that in the wild-type strain, particularly in fructose. The growth rate of the *trx1Δ trx2Δ* mutant was lower on the MS300 growth curves that measured optical density (Fig. 5C), which coincided with the data presented in Fig. 1A after counting of viable cells. This confirmed the impact of the mutation during fermentation as soon as growth started. To further analyze the effect of cytosolic thioredoxin deletion on fermentation independently of growth, fermentative capacity was measured as CO₂ production (Fig. 5D) in synthetic must

FIG 4 Legend (Continued)

ones in green. The metabolites in gray were not detected by the metabolome analysis. Five replicas of each strain were tested in the metabolome analysis, and three replicas were used for the proteome analysis. The levels of the relevant metabolites are shown as box diagrams.

FIG 6 Hexokinase activity remains constant despite thioredoxin deletion in grape juice fermentation. Total hexokinase activity was assayed under different growth conditions (YPD and MS300) in exponential (exp) and stationary (st) phases in the parental C9 strain and the *trx1Δ trx2Δ* double mutant. Activity data were normalized by total protein. Experiments were done in triplicate. Means and standard deviations are provided.

MS300. The *trx1Δ trx2Δ* mutant displayed less fermentative capacity per cell than the wild type, probably due to its lower amount of glycolytic enzymes. Therefore, this physiological impact at the hexose metabolism level was present in many different growth media and under many conditions, suggesting this redox control system's global implication in carbon metabolism under fermentative conditions.

As the protein levels of the three different hexokinases were affected in the thioredoxin mutant and they were the key control points of glycolysis, hexokinase activity was measured in YPD and synthetic grape juice MS300 in both the exponential and stationary phases (Fig. 6). Unexpectedly, the *trx1Δ trx2Δ* double mutant displayed increased hexokinase activity when cells grew exponentially under standard laboratory growth conditions. This was quite intriguing because this mutant showed delayed growth under the same condition (Fig. 5A). As cells were taken at the same optical density, it would seem that the increase in hexokinase activity could be a feedback mechanism that reflected a problem further down the metabolic pathway. The difference persisted when cells reached the stationary phase in the same medium although both activities diminished, thus indicating a regulation of these enzymes by the growth phase and probably reflecting cells reaching a postdiauxic state. The exponential growth in synthetic grape juice MS300 rendered similar levels of hexokinase activity but no differences between strains. Activities diminished in the stationary phase but, once again, no significant differences were found between the wild-type strain and the *trx1Δ trx2Δ* double mutant. As protein levels lowered in the *trx1Δ trx2Δ* strain for this condition (see Table S1 in the supplemental material), additional posttranslational activation of the enzyme could take place as a compensatory mechanism.

Some glycolytic enzymes undergo aggregation processes under specific environmental conditions like hypoxia and starvation (23, 24). One such enzyme was the main pyruvate kinase Cdc19p (25). As the Cdc19p level sharply dropped in the *trx1Δ trx2Δ* mutant (5.57-fold), the gene was green fluorescent protein (GFP)-tagged to analyze if aggregation could influence the level or potential activity of the enzyme (Fig. 7). After 6 h of exponential growth in the rich YPD medium in the absence of stress, Cdc19-GFP occupied the cytoplasm of the wild-type strain homogeneously, and only 4% of cells presented protein aggregation. However, the *trx1Δ trx2Δ* strain showed the presence of aggregates in one-third of cells under the same conditions. Fluorescence intensity was also lower in the mutant, which suggests a diminished amount of enzyme in the laboratory medium. These facts could explain the slower growth observed in YPD (Fig. 5A), as the enzyme in these aggregates is believed to be inactive (25). Aggregation concentrated in a single focus per cell, a pattern observed in this protein when reaching

FIG 7 Thioredoxin deletion increases Cdc19p aggregation in the exponential phase. (A) The green fluorescence and phase contrast images of the wild type and *trx1Δ trx2Δ* double mutant carrying a Cdc19p-GFP fusion. Samples were taken at exponential growth (6 h) in YPD and synthetic must and after 1 day in synthetic must. (B) Percentage of cells containing foci.

the stationary phase (25), while carbon starvation caused many smaller foci to form per cell. The same trend was observed after 6 h in synthetic grape juice MS300, with foci present in 26% of cells in the mutant strain, but, in this case, the difference with the wild type was slightly smaller. Foci were unique in both strains and media, which suggests similar formation modes. After a 1-day fermentation in synthetic grape juice, the amounts of cells with granules were similar (about one-fourth of them) in both strains. When cells reached their maximum cell density on day 3 for the parental strain and on day 6 for the mutant strain, almost all of the cells had Cdc19p aggregates (see Fig. S2 in the supplemental material). Hence, these aggregations seemed to be related to the stationary phase and, although these aggregations were present in both strains when the proteomic analysis was performed, their earlier establishment in the thiore-

doxin mutant could have influenced the amount of protein actively present in later growth stages, which would potentially affect glycolytic pathway activity.

DISCUSSION

The yeast cytoplasmic thioredoxin system is formed by two thioredoxins, Trx1p and Trx2p. This work analyzed their roles during wine making by deleting both thioredoxin genes. During grape juice fermentation in both synthetic and natural grape juice fermentations, the *trx1Δ trx2Δ* double mutant displayed slower growth (Fig. 1A) and a lower sugar consumption rate (Fig. 1C) but an extended CLS (Fig. 1B). The CLS is measured as the viability of nondividing cells with time. Under laboratory conditions, this state is reached when sugars are consumed, but under wine-making conditions, carbon is not the limiting nutrient and nitrogen usually is, so cells stop dividing but are metabolically active (17). As the double thioredoxin deletion strain has a reduced sugar metabolism (Fig. 1C and 5D), the apparent CLS extension reflects a lower metabolic ratio, a factor that is usually associated with extended life span (26). The CLS under wine-making conditions shortens when sugars are fully consumed (8), but this is not the case for this mutant. Nevertheless, the *trx1Δ trx2Δ* mutant was able to finish fermentation with the same ethanol production as the wild type (Fig. 1D). *TRX2* overexpression did not improve fermentative metabolism (see Fig. S2 in the supplemental material). This was probably due to one of several reasons. If these proteins were not limiting during grape juice fermentation, their increment without upregulation of the upstream (thioredoxin reductase) or downstream (peroxiredoxins) elements in the thioredoxin system would not be useful or could even have a deleterious effect. That would compensate for the potential positive effects seen in other respiratory situations, such as biomass production.

The defect of growth of the thioredoxin mutant in different media (Fig. 1 and 5) could be caused by a variety of cellular processes. The most obvious one would be the downregulation of translation detected by the proteomic analysis, which caused a defect in protein synthesis as confirmed by increased sensitivity to cycloheximide (Fig. 2B). Another affected factor could be the prolongation of the cell cycle S phase of the double thioredoxin mutants detected under laboratory conditions due to the inefficient reduction in ribonucleotide reductase (RNR), which is dependent on the cTRX system, leading to diminished deoxynucleoside triphosphate (dNTP) synthesis (12). Unfortunately, our metabolomic analysis did not detect dNTPs, and we were unable to fully establish the implication of the thioredoxin system in DNA synthesis for this growth condition.

The most obvious effect of thioredoxin deletion was a change in the cell's redox status, as reflected by the increased level of oxidized glutathione (GSSG) and the lower level of reduced glutathione (GSH) during fermentation (Fig. 3B). A conspicuous increase in the amount of Ecm4p, a glutathione transferase of the omega class, likely contributed to the drop in the GSH concentration. Besides, the increase in hydroxyl fatty acids (see Table S2 in the supplemental material) suggested enhanced oxidative damage. However, no increase at the protein level occurred in global terms in either the antioxidant enzymes or the enzymes involved in glutathione synthesis (see Table S1 in the supplemental material), which suggests that the remaining antioxidant systems were able to cope with oxidative stress with no further activation.

Striking effects of thioredoxin deletion at the fermentative metabolome level are reported herein. Diminished concentrations of phosphorylated hexoses in the *trx1Δ trx2Δ* double mutant were coincident with the marked decreases in the abundance of all three hexokinase isoforms (Hxk1p, Hxk2p, and Glk1p) detected at the proteome level. The double mutant strain's lowered fermentative capacity (Fig. 1C and 5D) and growth rate (Fig. 1A and 5C) under several growth conditions were, thus, explained at both the proteome and metabolome levels. Unexpectedly, no differences were found in hexokinase activity in the cell extracts from synthetic must-fermenting cells (Fig. 6). However, it is not uncommon to find discrepancies between *in vivo* data for enzyme action and *in vitro* measurements of enzymatic activities by standard assays under

optimal conditions, due mainly to different chemical compositions and molecular connections. This scenario suggests that some feedback regulation was triggered by thioredoxin deletion. *TRX2* overexpression increased hexokinase activity during biomass propagation (14), which indicates that the regulation of the first hexose metabolism step was sensitive to the status of the cytosolic thioredoxin system. These data revealed that less glucose entered the glycolytic pathway in the double mutant than in the wild-type strain, while the net metabolic flux down to pyruvate did not change. Two facts could explain this apparently contradictory result. First, the metabolic flux diversion toward the lateral pathways could diminish in the mutant. Second, the high levels of F1-6BF, a known positive allosteric effector of pyruvate kinase, in the mutant strain could result in increased activity of this enzyme despite its lower abundance, which could contribute to sustaining the global glycolytic flux. Hxk2p is a key controller of glucose repression, but the *trx1Δ trx2Δ* double mutant displayed no altered sensitivity to 2-deoxyglucose, a glucose analog that triggers glucose repression (data not shown). Alternatively, the oxidative damage of membrane lipids could impact sugar transport by contributing to changes in global carbon metabolism.

The differences in the metabolic protein amount detected by the proteomic analysis could be influenced by protein stability and solubility. We determined that thioredoxins influenced the aggregation of pyruvate kinase Cdc19p in foci, which has been related to stress granules and also to cytosolic accumulation where proteins are sequestered during stress (25). The *trx1Δ trx2Δ* double mutant showed aggregation for this protein early during must fermentation (Fig. 7), which could influence metabolism from the very beginning. The aggregation of glycolytic enzymes during hypoxia has been related to the presence of ROS produced by mitochondria (23). *HSP31* deficiency has been demonstrated to increase the ROS generated by mitochondria (27), and it has been shown that Hsp31p acts as a chaperone preventing protein aggregation (28). Here, we showed that Hsp31p was strongly downregulated in the *trx1Δ trx2Δ* double mutant, which could explain the enhanced propensity to the aggregation of Cdc19p. Interestingly, in such aggregates from a wild-type strain, and together with glycolytic enzymes, both Trx1p and Trx2p have been isolated (23). Thioredoxin depletion may enhance this event. Cdc19p aggregation also interferes with the localization of transcription factor Sfp1 (25), probably by TOR inhibition. Sfp1p regulates ribosome biogenesis. Yeast extract (22) indicates that 93.56% of the proteins differentially expressed in the thioredoxin mutant are regulated by this transcription factor. Therefore, a defective transcriptional regulation set from the very beginning of fermentation could cause the differences found at the protein levels, which could alter metabolism in the long term. This could also influence the impact of cTRX on protein biosynthesis. The study of early time points would reveal if such a mechanism had operated. Fermentative metabolism and redox control seemed very robust, so there could be other systems that operated in both mitochondria and cytosol to compensate for thioredoxin loss. The translation machinery was downregulated in the mutant strain, and many amino acid biosynthetic and aminoacyl-tRNA synthetase enzymes decreased (Table S1). This could well be the cause of the strain's delayed growth and may suggest a regulation of pathways such as TORC1, whose activity promotes translation and growth. However, the mutant was not sensitive to TORC1 inhibitor rapamycin (data not shown); thus, potential regulation could have occurred at a lower level.

We also demonstrated that the deletion of the cTRX system impacted lipid metabolism (Fig. 3) by increasing desaturase Ole1p and the amount of unsaturated fatty acids. The growth defect could cause fatty acids to accumulate, which would result in the amount of enzymes Fas1p and Fas2p, involved in fatty acid synthesis, being inhibited. Low levels of free fatty acids (apart from hydroxyl fatty acids) could reflect low activity of phospholipase. Actually, lysophospholipase 1, Plb1p (P39105), was highly repressed (10.8-fold) in the double mutant strain (Table S1), which would explain the abundance of phospholipids and low levels of free fatty acids. High levels of stearate and arachidate could be due to the action of other active phospholipases specific for long-chain fatty acids. Sphingolipid responsive proteins Pil1p and Lsp1p were also downregulated

in the mutant strain, which could be related to the elevated levels of sphingolipids. Alternatively, as Fas1p and Fas2p have been found in the same granules as Cdc19p (23), a more direct mechanism of regulation could take place in this case. Ergosterol also accumulated (Fig. 3B), but no change in the enzymes involved in the ergosterol biosynthetic pathway was noted. Together with Ole1p, other proteins were more abundant in the *trx1Δ trx2Δ* strain and, despite the fermentative conditions, were related to respiratory metabolism and mitochondria (Fig. 2C and Table S1). No clear pathway emerged from the regulation of this set of proteins under these conditions, so it could rely on a specific mechanism for this fermentative situation.

MATERIALS AND METHODS

Yeast strains and growth media. The yeast strains used in this work were derived from the C9 strain (16). To perform gene disruptions, recyclable selection marker *loxP-kanMX-loxP* from plasmid pUG6 (29) and recombinase *cre* under an inducible *GAL* promoter (30) were used to excise the *kanMX* marker. The GFP tagging of *CDC19* was done with plasmid pFA6-GFP-KanMX6 (31). Multicopy plasmid Yep-TRX2 (13) was used to overexpress *TRX2*. Yeast transformations were performed by the lithium acetate method (32). Yeasts were usually grown in rich YPD medium (1% yeast extract, 2% Bacto peptone, 2% glucose). Solid plates contained 2% agar and 20 $\mu\text{g ml}^{-1}$ G418 (Geneticin; ThermoFisher) if required. Synthetic grape juice MS300 was made as previously described (33). Red grape juice (Bobal variety) was a gift from Bodegas Murviedro (Requena, Spain). Cycloheximide resistance was measured by spreading on YPD plates the equivalent of 2 OD₆₀₀ units of a stationary culture in YPD of the strain of interest and placing 5 μl of 1 mM cycloheximide in a circle of paper in the middle of the plate. The inhibition halo was measured after incubating the plates for 1 day.

Natural and synthetic must fermentation, growth curves, fermentative capacity measurement, and microscopy methods. The fermentation experiments were conducted in both natural and synthetic grape juices with the cells from stationary cultures in YPD inoculated at a final concentration of 10⁶ cells/ml in filled-in conical centrifuge tubes with 30 ml of must. Incubation was done with low shaking at 25°C. The vinification progress was followed by determining CFU (by serial dilution, plating, and colony counts) and sugar consumption using a reaction to DNS (3,5-dinitrosalicylic acid) (34). Growth curves were performed in a Varioskan Lux plate reader at 30°C with shaking. To measure fermentative capacity, precultures were grown overnight on YPD, inoculated at a final concentration of 10⁷ cells/ml in synthetic must, and incubated at 30°C. CO₂ production was measured every 10 min by an Ankom RF gas production system. Fermentative capacity was expressed as milliliters of CO₂ produced per 10⁷ cells. The GFP-labeled cells were directly observed in the synthetic grape juice fermentations. The cells grown in YPD were washed in 1× phosphate-buffered saline (PBS) before visualization. Cells were visualized with the right filter under a Nikon Eclipse 90i fluorescence microscope.

Proteomic analysis. Three cultures of the C9 and C9 *trx1Δ trx2Δ* mutant strains were grown in MS300 synthetic must, and cell samples were taken in the stationary phase when cultures reached maximum cell density at 3 and 6 days, respectively. The remaining sugar concentration was around 50 g/liter. We followed the protocol described by McDonagh et al. to prepare protein samples (35, 36). Cells were harvested, and the pellet was washed twice with water and redissolved in lysis buffer (8 M urea, 100 mM *N*-ethylmaleimide [NEM], 50 mM Tris-Cl, 2 mM EDTA, 0.1% [vol/vol] Triton X-100, and 2 mM phenylmethylsulfonyl fluoride [PMSF], pH 8.0) containing washed sea sand. Cells were washed twice with 50 mM bicarbonate buffer containing 50 mM *N*-ethylmaleimide to alkylate Cys with their reduced sulfhydryl group, and finally the pellet was resuspended in the same solution with 0.1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}. Cells were lysed through cryogenic homogenization with liquid nitrogen. Samples were centrifuged at 15,000 × *g* for 10 min at 4°C to obtain protein extracts. Three independent cultures were used per strain. Samples were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The LTQ Orbitrap operated in the parallel mode with a resolution setting at 30,000 of width at half height within the 400 to 1,500 *m/z* range, followed by five collision-induced dissociation activated MS/MS in the DDA (data-dependent acquisition) mode to select the most abundant masses. The maximum injection times for mass spectrometry (MS) and MS/MS were 500 ms and 50 ms, respectively. Monoisotopic precursors were selected by excluding the species with charge +1, and normalized collision energies of 35% for CID (collision induced dissociation) were used. The minimum intensity threshold for MS/MS was 1,000 counts. MS/MS spectra were analyzed with the SEQUEST search engine against the UniProt database of *S. cerevisiae* (www.uniprot.org). The software for “label-free” quantification Progenesis Q1 for proteomics (Nonlinear Dynamics, Waters) was used, along with the digital image files that contained all of the data of the image captured by the sensor (RAW). Three RAW files were used per sample from three separate experiments. The peptide signals represented in the retention time versus the *m/z* ratio were aligned and quantified from the area under the curve (AUC) to produce a list of peaks, which were grouped and identified with the Proteome Discoverer search engine (v 1.4) against the UniProt database. *P* values were generated in Progenesis Q1 using analysis of variance (ANOVA), where significant differences were determined based on a *P* value of <0.05.

Metabolomic analysis. Five cultures of the C9 and C9 *trx1Δ trx2Δ* mutant strains were grown in MS300 synthetic must and were taken in the stationary phase under the same conditions as the above-indicated proteomic analysis. Cell pellets were frozen and analyzed by Metabolon Inc. (Durham, NC, USA). Samples were prepared in the MicroLab STAR system (Hamilton Company). The resulting

extract was divided into four fractions as follows: two for the analysis by two separate reverse-phase/ultraperformance liquid chromatography (RP/UPLC)-MS/MS methods with positive ion mode electrospray ionization (ESI), one for the RP/UPLC-MS/MS analysis with the negative ion mode ESI, and one for the hydrophilic interaction liquid chromatography (HILIC)/UPLC-MS/MS analysis with the negative ion mode ESI. A PCA was performed in Array Studio. Each principal component was a linear combination of every metabolite, and principal components were uncorrelated. The number of principal components equaled the number of observations.

Determination of hexokinase activity. The cells grown in the different media were collected by centrifugation and then broken with glass beads in extraction buffer (50 mM phosphate buffer at pH 7.4, 2 mM EDTA, 2 mM β -mercaptoethanol) (37). Hexokinase activity was assayed by NADPH formation, determined at 340 nm in a 1-ml cuvette containing 10 mM glucose, 20 mM Tris-HCl buffer, pH 7.5, 7.5 mM $MgCl_2$, 0.5 mM ATP, 0.65 mM $NADP^+$, 2 units of glucose-6-phosphate dehydrogenase, and 10 to 50 μ l of a suitable diluted enzyme preparation. Total protein was quantified by the Bradford method (Bio Rad).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.02953-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.1 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.3 MB.

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C.P., J.A.B., E.M., and A.A. conceived and designed the experiments; C.P., J.P., and A.A. performed the experiments; C.P., B.M., J.A.B., E.M., and A.A. analyzed the data; J.A.B., E.M., and A.A. contributed reagents, materials, and analysis tools; and C.P. and A.A. wrote the paper.

We declare no conflicts of interest.

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