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Physiological and Transcriptional Responses to High Concentrations of Lactic Acid in Anaerobic Chemostat Cultures of *Saccharomyces cerevisiae*[▽]

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Based on the high acid tolerance and the simple nutritional requirements of *Saccharomyces cerevisiae*, engineered strains of this yeast are considered biocatalysts for industrial production of high-purity undissociated lactic acid. However, high concentrations of lactic acid are toxic to *S. cerevisiae*, thus limiting its growth and product formation. Physiological and transcriptional responses to high concentrations of lactic acid were studied in anaerobic, glucose-limited chemostat cultures grown at different pH values and lactic acid concentrations, resulting in a 50% decrease in the biomass yield. At pH 5, the yield decrease was caused mostly by osmotically induced glycerol production and not by the classic weak-acid action, as was observed at pH 3. Cultures grown at pH 5 with 900 mM lactic acid revealed an upregulation of many genes involved in iron homeostasis, indicating that iron chelation occurred at high concentrations of dissociated lactic acid. Chemostat cultivation at pH 3 with 500 mM lactate, resulting in lower anion concentrations, showed an alleviation of this iron homeostasis response. Six of the 10 known targets of the transcriptional regulator Haa1p were strongly upregulated in lactate-challenged cultures at pH 3 but showed only moderate induction by high lactate concentrations at pH 5. Moreover, the *haa1*Δ mutant exhibited a growth defect at high lactic acid concentrations at pH 3. These results indicate that iron homeostasis plays a major role in the response of *S. cerevisiae* to high lactate concentrations, whereas the Haa1p regulon is involved primarily in the response to high concentrations of undissociated lactic acid.

Lactic acid has traditionally been used in many industrial applications, including food preservation and production of cosmetics and pharmaceuticals (5). Today, concerns for environmental issues and oil availability contribute to an increased interest in polylactic acid as a bioplastic produced from renewable feedstocks (11). Lactic acid is currently produced predominantly with various species of lactic acid bacteria (5). Due to their complex nutritional requirements and sensitivity to low pH, these bacteria are suboptimal for the production of the high-purity, undissociated lactic acid (pK_a = 3.86 [12]) required for polylactic acid production (5, 10).

Engineered *Saccharomyces cerevisiae* strains are under evaluation as possible alternative lactic acid producers. Deletion of one or more of the functional genes encoding pyruvate decarboxylase in combination with the expression of a heterologous lactate dehydrogenase has resulted in *S. cerevisiae* strains with reduced or eliminated ethanol formation that are capable of producing L-lactic acid (3, 26, 46, 50, 60). The comparatively high acid tolerance of *S. cerevisiae* and its simple nutritional requirements should facilitate the production of undissociated lactic acid instead of the lactate anion that is formed at a higher pH in bacterial fermentations.

In the food industry, lactic acid is commonly used as an acidulant and a preservative (6, 54). In comparison with other weak acid preservatives, *S. cerevisiae* is relatively insensitive to lactate, and strong inhibitory effects require relatively high lactate concentrations (1, 18, 40, 52). As has been demonstrated for several other weak organic acid preservatives, the mechanism by which food is preserved by lactic acid is thought to be based, at least to some degree, on the intracellular accumulation of protons mediated by the diffusion of undissociated acid into the cells (14, 39). Although the inhibitory actions of lactic acid on yeast growth and metabolism are pH dependent and are accompanied by changes in the intracellular pH, there are indications that the mechanism underlying its toxicity differs from that of other weak acid preservatives (39) and involves the toxicity of the lactate ion (56). Based on transcriptional regulation studies, genes pertaining to cell wall architecture and a set of genes controlled by the transcriptional regulator Aft1p, which are involved in iron uptake and metabolism, have been implicated in resistance to lactic acid (29). However, phenotypic screening of deletion mutants in genes pertaining to iron homeostasis did not reveal an increased sensitivity to lactic acid or acetic acid (29).

Lactic acid toxicity at high concentrations or low pH (as required for the production of free acid) is likely to represent a major challenge for the industrial production of lactic acid, where very high concentrations of free lactic acid are desired. However, lactate toxicity and tolerance in *S. cerevisiae* are not only relevant for industrial lactate production. In addition, lactic acid is commonly found in industrial yeast fermentations, where the proliferation of contaminant lactic acid bacteria leads to lactic acid accumulation (38, 40). Furthermore, the presence of lactic acid in combination with other stressors has been shown to synergistically inhibit yeast growth and metabolism (52) and, thus, to affect many yeast-based industrial fermentations (40).

The aim of the present study was to analyze the physiological

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and transcriptional responses of *S. cerevisiae* to lactic acid stress. To dissect the organism's responses to lactic acid from those to the lactate anion, experiments were performed at pH 3 and pH 5. Anaerobic, glucose-limited chemostat cultures were used to enable a quantitative comparison of the transcriptional regulation and physiological effects of lactic acid at a fixed specific growth rate. To further facilitate the comparison, the concentrations of lactic acid used in the chemostat cultivation experiments were chosen such that they resulted in the same reduction of the biomass yield on glucose at both pH 3 and 5.

MATERIALS AND METHODS

Chemostat growth conditions. The laboratory reference strain CEN.PK 113-7D (*MAT***a**) was grown at 30°C in 1.5-liter chemostat fermentors (Applikon, Schiedam, The Netherlands). A comparable degree of weak acid stress was ensured by decreasing the biomass yield to approximately 50% of the reference condition (no added lactic acid) with the addition of the appropriate amount of L-lactic acid to the medium vessel prior to sterilization. All cultures, including that of the reference strain, were fed with synthetic medium as described by Verduyn et al. (61), with 25 g liter^{-1} glucose as the limiting nutrient and 0.15 ml liter^{-1} silicone antifoam (BDH, Poole, England) to prevent excessive foaming. The dilution rate was set to 0.10 h^{-1}, and the pH was controlled at 5.0 or 3.0 with the automatic addition (ADI 1031 bio controller; Applikon) of 2 M KOH. The stirrer speed was set at 800 rpm, and anaerobicity was maintained by sparging the fermentor with N_2 gas at 500 ml min^{-1}. To prevent diffusion of oxygen, the fermentor was equipped with Norprene tubing and Viton O-rings, and the medium vessel was also flushed with N_2 gas.

Analytical methods. Chemostat cultures were assumed to be in at steady state when, after at least five volume changes, the culture dry weight and specific carbon dioxide production rate changed by less than 2% over two volume changes. Steady-state samples were taken between 10 and 14 volume changes after inoculation to avoid possible evolutionary adaptation during long-term cultivation. Culture dry weight measures were determined in duplicate via filtration onto dry, preweighed nitrocellulose membranes. Samples were dried in a microwave oven for 20 min at 360 W. Culture supernatants were obtained by centrifugation of the chemostat broth or by a rapid sampling method using precooled (−20°C) steel beads (34). For the purpose of flux determination and carbon recovery, supernatants and media were analyzed via high-performance liquid chromatography using an Aminex HPX-87H ion exchange column with 5 mM H_2SO_4 as the mobile phase. Off gas was first cooled with a condenser (2°C) and then dried with a Perma Pure dryer (model no. PD-625-12P). CO_2 and O_2 concentrations in the off gas were measured with an NGA 2000 Rosemount gas analyzer.

Microarray analysis. Sampling of chemostat cultures at pH 5 was performed by instantly quenching the yeast culture in liquid nitrogen, as described previously (44). However, in contrast to sampling cultures at pH 5, for which the protocol was optimized, sampling cultures at pH 3, especially in the presence of high lactic acid concentrations, with this method did not yield sufficient quantities of mRNA to proceed with cDNA synthesis. Therefore, pH 3 cultures were quenched in ice-cold Tris-EDTA (TE) buffer at pH 8 (using 5× the volume of sample) and then washed in ice-cold TE buffer (with 2× the volume), followed by a wash in ice-cold demineralized water (with 2× the volume). Finally, cells were resuspended in acetate-EDTA buffer, sodium dodecyl sulfate, and acid phenol-chloroform as previously described (44). Probe preparation and hybridization to Affymetrix GeneChip microarrays were performed as described previously (2).

Data acquisition, quantification of array images, and data filtering were performed with Affymetrix Microarray Suite version 5.0, MicroDB version 3.0, and Data Mining Tool version 3.0. All arrays were scaled by normalizing the average signal from all probes to a value of 150. Since transcripts with values below 12 cannot be measured accurately, their levels were set to 12 for statistical analysis (44). Groups of genes which were up- or downregulated in the presence of lactic acid at pH 3 or pH 5 were examined for enrichment, using MIPS database functional annotation (35) and significant transcription factor binding, as previously described (2). To enable further study of these data by other researchers, the data from Affymetrix GeneChip microarrays used in this study are available via Gene Expression Omnibus series accession number GSE10066 (http://www.ncbi.nlm.nih.gov/projects/geo/query/acc.cgi?acc=GSE10066).

Strain construction. Gene deletions were generated in the genetic background of the prototrophic strain CEN.PK 113-7D by using standard yeast media and genetic techniques (8). The *Kan*MX marker was amplified by using pUG6 as a template (19) and specific primers. The resulting disruption cassettes, containing sequences homologous to the targeted genes, were transformed using the protocol for high-efficiency transformation of yeasts (8). After recovering, the cells were plated on YPD medium containing G418 (200 μg/ml). Confirmation of successful gene disruptions was performed using colony PCR.

To generate the *tpo2 tpo3* double deletion, the *hph*NT1 marker was amplified using pFA6a-*hph*NT1(27) as a template and specific primers targeting *TPO3*. The *TPO3* disruption cassette was transformed into the *tpo2*Δ strain, and transformants were selected on YPD medium containing 20 μg/ml hygromycin B. Deletion of both *TPO2* and *TPO3* was confirmed by colony PCR.

Anaerobic batch cultivation for phenotypic screening. The preinoculum for the anaerobic batch cultures was produced by performing an anaerobic chemostat for each strain at pH 3 and bringing it to steady state (the medium and conditions were as described above, with no lactic acid added). The fermentor was then emptied until only approximately 50 ml remained as the inoculum for batch growth. Then, fresh medium containing 500 mM lactic acid was added, and batch fermentation was initiated. Specific growth rates in the batch phase were calculated based on continuous off-gas CO_2 measurements (as described above). Upon completion of the batch culture (depletion of glucose), the fermentor was once again emptied and refilled with fresh medium without lactic acid, and chemostat cultivation resumed until steady state was established at a dilution rate of 0.1 h^{-1}. Then, the fermentor was emptied and refilled once more, and growth rates were determined for each strain in an anaerobic batch culture containing 750 mM lactic acid at pH 3 (as described above for 500 mM lactic acid).

RESULTS

Physiological responses to lactic acid. Chemostat cultivation makes it possible to study the effect of environmental stimuli at a fixed specific growth rate. Therefore, quantitative comparisons of the physiological effects and transcriptional responses to lactic acid at different pH values were performed with anaerobic, glucose-limited chemostat cultures. Anaerobic conditions were utilized to prevent lactate consumption via respiratory metabolism (33). To this end, experiments were performed in which the concentration of lactic acid in the medium was titrated to reduce the biomass yield on glucose to approximately 50% of the biomass yield in anaerobic, glucose-limited reference cultures (no lactic acid added) at each pH value.

For cultures grown at pH 5, 900 mM of lactic acid (~61 mM undissociated acid) was required to decrease the biomass yield on glucose to 50% of the biomass yield in reference cultures (Fig. 1). Assuming that the undissociated species determines weak organic acid toxicity, the Henderson-Hasselbach equation (assuming a pK_a value of 3.86 for lactic acid [12]) can be used to estimate the concentration of lactic acid required to obtain a similar yield reduction at pH 3.5. This led to the prediction that a lactic acid concentration of 85 mM (~60 mM undissociated acid at pH 3.5) should cause a 50% reduction of the biomass yield at pH 3.5 (Fig. 1). However, experiments showed that the required concentration was almost 9-fold higher (750 mM) (Fig. 1). Further experiments at pH 3 showed that, even at this low pH, 500 mM lactic acid was required to reduce the biomass yield to approximately 50% of that of the reference condition (Table 1). In contrast, benzoic acid at total concentrations of 2 mM (pH 5) and 0.3 mM (pH 3.5), corresponding to 0.27 mM and 0.25 mM undissociated acid, respectively, showed the same degree of reduction of the biomass yield, thus confirming that for benzoic acid, toxicity is mediated predominantly by the undissociated species.

Using lactate concentrations that resulted in approximately

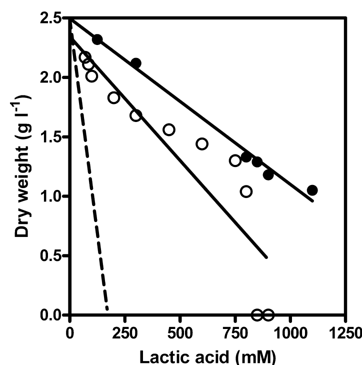

FIG. 1. Effect of lactic acid on biomass formation in glucose-limited anaerobic chemostat cultures of *S. cerevisiae* CEN.PK 113-7D. Each data point represents an independent chemostat culture which was grown to steady state at a dilution rate of 0.10 h^{-1}. Based on the data obtained at pH 5.0 (●), the Henderson-Hasselbach equation was used to estimate an equivalent stress at pH 3.5 (dashed line), assuming that the undissociated acid was solely responsible for the observed decrease of the biomass concentration. Experimental data obtained at pH 3.5 (○) did not correlate with this prediction.

50% reduction of the biomass yield, triplicate anaerobic chemostat fermentations were performed at pH 3 and 5, and results were compared to those of reference fermentations without lactic acid. Steady-state fluxes and carbon recoveries were calculated from substrate and metabolite concentrations in growth media and culture supernatants (Table 1).

Residual concentrations of glucose in cultures grown in the presence of lactic acid were higher than those in the reference cultures. In microorganisms, the specific rate of consumption of the growth-limiting substrate, q_s, often exhibits saturation kinetics with respect to its concentration, C_s (36). These kinetics can be described by the modified Monod equation,

$$q_s = q_s^{\max} \frac{C_s}{C_s + K_s}$$

Thus, the increased rate of glucose consumption by the cultures may be at least partially responsible for the increased residual glucose concentration. However, especially in the chemostat cultures grown at pH 3, this phenomenon cannot fully account for the drastic increase of the residual glucose concentration in cultures grown with lactic acid. This suggests that lactic acid alters the kinetics of glucose transport, for example, by altering the expression or kinetic parameters (K_s or $V_{\max}$) of plasma-membrane glucose transporters (56) or by perturbation of the plasma membrane structure (9). Since the residual glucose concentrations remained well below 5 mM in the cultures at pH 5, no substantial impact of glucose repression on gene expression was anticipated (62), nor was it observed in the current study. At pH 3 in the presence of lactate, the residual glucose concentration was elevated to levels that have previously been reported to cause glucose repression, but extensive downregulation of glucose-repressible genes was not observed.

Specific rates of glycerol production were higher for cultures challenged with lactate than for reference cultures. The differences were most notable at pH 5, where the high concentration of (dissociated) lactic acid, combined with the large amounts of KOH required to achieve pH 5 after lactic acid was added to the medium, resulted in a dramatically increased salt concentration. As glycerol is a well-known compatible solute that counteracts osmotic pressure in yeast cells (22), the elevated levels of glycerol were likely an artifact caused by the presence of the high salt concentrations. Conversion of 0.5 mol glucose into 1 mol of glycerol requires the input of 1 mol of ATP. Under anaerobic conditions, this ATP has to be provided by the dissimilation of an additional 0.5 mol of glucose through glycolysis. In cultures grown at pH 5, the increase in glucose consumption for glycerol production could largely account for the major part of the increased specific rate of glucose consumption and, hence, for the decreased biomass yield on glucose (Table 1). Glycerol production was less pronounced in lactate-challenged cultures grown at pH 3, to which less lactic acid and, especially, less KOH were added (Table 1). Under these conditions, only a small part of the observed decrease of the biomass yield could be attributed to glycerol production.

TABLE 1. Physiological analysis of chemostat cultures of *S. cerevisiae* in the presence and absence of high lactate concentrations[a]

Physiological parameter (units)	Avg value ± SD			
	pH 5		pH 3	
	Reference (no acid)	900 mM lactic acid	Reference (no acid)	500 mM lactic acid
Glucose (mmol g^{-1} h^{-1})	−6.03 ± 0.10	−11.46 ± 0.31	−6.96 ± 0.39	−10.32 ± 0.37
CO_2 (mmol g^{-1} h^{-1})	10.40 ± 0.45	17.63 ± 0.85	11.76 ± 0.29	18.02 ± 0.32
Ethanol (mmol g^{-1} h^{-1})	9.52 ± 0.16	13.13 ± 0.27	10.99 ± 0.43	17.56 ± 0.74
Glycerol (mmol g^{-1} h^{-1})	0.79 ± 0.02	5.06 ± 0.41	0.83 ± 0.04	1.90 ± 0.07
Lactate (mmol g^{-1} h^{-1})	0.05 ± 0.01	ND[b]	0.09 ± 0.01	ND
Acetate (mmol g^{-1} h^{-1})	0.02 ± 0.00	0.72 ± 0.09	0.02 ± 0.00	0.10 ± 0.01
Biomass (g $liter^{-1}$)	2.25 ± 0.02	1.21 ± 0.02	2.03 ± 0.04	1.21 ± 0.04
Biomass yield (g [g glucose]$^{-1}$)	0.09 ± 0.00	0.05 ± 0.00	0.09 ± 0.00	0.05 ± 0.01
Carbon recovery (%)[c]	99.4 ± 0.8	93.1 ± 2.5	96.46 ± 3.1	98.52 ± 3.3
Residual glucose (mM)	0.2 ± 0.0	3.0 ± 0.8	0.4 ± 0.1	10.3 ± 1.3

[a] Physiology of anaerobic, glucose-limited, chemostat cultures of *S. cerevisiae* (dilution rate, 0.10 h^{-1}) grown in the presence and absence of high lactate concentrations at pH 5 and pH 3. Lactate concentrations were chosen such that they resulted in an approximately 50% decrease of the biomass yield relative to that of the reference condition (Fig. 1). Specific rates of glucose consumption and product formation and other parameters are represented as averages ± standard deviations (SD) for three independent cultures for each condition. Due to the high concentrations of lactic acid added to these cultivations, the production or consumption rates of lactic acid could not be determined accurately. For the same reason, lactate was not included in calculations of carbon recovery for these conditions.

[b] ND, not determined.

[c] Acetaldehyde fluxes were not included in the determination of carbon recovery as acetaldehyde measurements were performed only for cultures at pH 5 with lactic acid.

TABLE 2. Transcriptional responses to high concentrations of lactic acid[a]

Category	No. of genes at:	
	pH 3	pH 5
Unexpressed	1,153	846
Unchanged	4,942	4,881
Common upregulated	51	51
Specific upregulated	50	296
Total upregulated	101	347
Common downregulated	95	95
Specific downregulated	92	214
Total downregulated	187	309

[a] Overall transcriptional response to high concentrations of lactic acid at pH 3 (500 mM lactic acid) and pH 5 (900 mM lactic acid). A twofold change with a false-discovery rate of 0.5% was applied as the selection criterion to identify significantly changed transcripts (Total), which were broken down into common or specific responses. Cultures grown at the respective pH in the absence of lactic acid were used as the baseline for comparison. The "common" category is representative of transcripts which were up- or downregulated at both pH 3 and 5. The "specific" category represents transcripts that were identified as being significantly changed at only one pH.

The increased acetate flux in lactate-challenged cultures is probably linked directly to the elevated glycerol production. Glycerol production leads to increased formation of NAD^+ (59), which can be balanced by the formation of oxidized products such as acetate. Consistent with this interpretation, the increased acetate production was less pronounced at pH 3 (Table 1). Carbon recovery in lactate-challenged cultures grown at pH 5 was only 93% (Table 1). With a CO_2 production rate that was higher than that expected from growth, ethanol and acetate formation, and a gap in the redox balance, the possible formation and evolution of acetaldehyde were investigated. Acetaldehyde formation results in the net production of 1 mol of NADH per mole and, thus, represents another means of balancing the NADH requirement for glycerol production in response to osmotic stress. Consequently, off-gas trapping was performed with 0.5 M TRIS-HCl (pH 9.0) (15, 37), and a flux of approximately 1.0 mmol acetaldehyde g^{-1} h^{-1} was observed. This represents a 2.4% increase in carbon recovery.

Transcriptional profiling: data quality and overall response. To obtain statistically reliable transcriptome data, triplicate chemostat cultures and oligonucleotide array analyses were carried out for lactic acid-challenged scenarios, as well as for the corresponding reference scenarios at pH 3 and pH 5. The average coefficient of variation for triplicate arrays under each condition was less than 18%. To allow for a comparison between the present data and that of previous chemostat-based transcriptome studies of other organic acids (2), a false-discovery rate of 0.5% with a twofold change was applied as the selection criterion used to identify significantly changed transcripts. A comparison of lactic acid-challenged cultures with those grown under the appropriate reference conditions revealed that in contrast to cultures grown at pH 3, cultures grown at pH 5 showed a larger number of transcripts that responded to lactic acid (Table 2). The role of the more strongly increased osmotic pressure at pH 5 in this response was substantiated by the upregulation of many genes involved in glycerol synthesis.

Approximately 2.3% of the entire genome (146 genes) showed a qualitatively similar response to lactate at pH 3 and pH 5. Of these consistently lactate-responsive genes, 51 genes were commonly upregulated and 95 downregulated at both pH values. Finally, a significant number of genes exhibited a response to lactic acid that was specific for one of the pH values (Table 2). Genes that shared the same qualitative transcriptional response (up or down) to lactate and/or culture pH were clustered in groups, which were analyzed in more detail and are discussed below.

pH-independent transcriptional responses. Comparisons of the overall transcriptional responses to lactate at pH 3 and pH 5 revealed major effects of culture pH. To identify pH-independent (commonly regulated) changes in transcript levels, two clusters of genes were defined that were consistently up- or downregulated at both pH 3 and 5. Genes that showed a transcriptional response to lactate only at pH 3 or at pH 5 were considered to be part of the pH-dependent lactate response (see below). The clusters thus identified were examined for enrichment of transcription factor binding (20) and MIPS functional categories (35).

Genes in the functional categories "siderophore iron transport" and "amine/polyamine transport" were significantly overrepresented among the genes that showed a pH-independent transcriptional upregulation in response to lactate (Table 3). The functional categories "metabolism" (including a number of subcategories), "energy," and "amino acid transport" were enriched in the set of commonly downregulated genes.

Genes that have been shown to bind the transcription factors Hap1p and Rcs1p/Aft2p were overrepresented in the set of genes that showed a pH-independent upregulation in lactate-challenged cultures, while only Stp1p/Bap1p was overrepresented among the downregulated genes (Table 4). Hap1p and Rcs1p/Aft2p are key determinants of iron homeostasis, and their identification in this gene set is corroborated by enrichment of the corresponding functional categories. Likewise, identification of enrichment for Stp1p/Bap1p binding in the pH-independent downregulated gene set can be linked directly to the downregulation of amino acid metabolism.

pH-dependent transcriptional responses. While the Rcs1p/Aft1p and Hap1p transcription factor binding sites were already significantly enriched in the set of genes that showed a pH-independent response to lactate (Table 4), genes involved in the homeostasis of iron and other metals were even more strongly overrepresented among the genes that showed a specific transcriptional upregulation at pH 5 (Table 3). Conversely, the genes that showed a transcriptional response to lactate only at pH 3 were not significantly enriched in any particular functional category (Table 3). This may reflect the fact that the lactate response at pH 5 is composed of the response to free lactic acid (as observed at pH 3), as well as additional responses related to the lactate anion.

Genes that are bound by the transcription factors Sko1p, Skn7p, and Cin5p were specifically overrepresented among the genes that were transcriptionally upregulated in lactate-challenged cultures grown at pH 5 (Table 4). These transcription factors are involved in the regulation of osmotolerance and salt tolerance, and their overrepresentation among these genes is probably a consequence of the experimental setup, which, at pH 5, involved high concentrations of lactate anions and po-

TABLE 3. MIPS functional categories overrepresented among lactic acid-responsive transcripts[a]

MIPS category	Significance ($\log_{10} P$ value)				
	Overall		Common	Specific	
	pH 3	pH 5		pH 3	pH 5
Metabolism	(−) 5.26	(−) 10.17	(−) 7.84		(−) 4.15
C compound and CHO metabolism	(−) 5.30	(−) 6.54	(−) 5.09		
C compound and CHO utilization	(−) 4.07	(−) 7.17	(−) 3.95		(−) 3.92
C compound and CHO catabolism		(−) 5.18			
Amino acid metabolism		(−) 5.97	(−) 3.72		
Metabolism of urea		(−) 4.13			
N and S utilization		(−) 4.22	(−) 3.78		
Energy	(−) 8.30	(−) 6.68	(−) 5.55		
Metabolism of energy reserves		(−) 4.54			
Transported compounds (substrate)		(+) 3.78/(−) 3.88			
Amino acid transport		(−) 4.55	(−) 4.44		
Amine/polyamine transport			(+) 3.79		
Allantoin/allantoate transport					(−) 4.11
Ion transport		(+) 6.41			(+) 5.23
Cation transport		(+) 5.59			(+) 4.78
Heavy metal ion transport		(+) 9.09			(+) 7.25
Siderophore iron transport		(+) 7.54	(+) 3.99		(+) 3.90
Cell rescue, defense, and virulence		(+) 3.82/(−) 4.17			
Ionic homeostasis		(+) 4.35			
Homeostasis of cations		(+) 4.64			
Homeostasis of metal ions		(+) 8.20			(+) 5.76

[a] Overview of MIPS functional categories overrepresented among lactic acid-responsive transcripts identified in a comparison of lactate-challenged and reference anaerobic chemostat cultures of *S. cerevisiae* CEN.PK 113-7D at pH 3 and pH 5. Overrepresentation is indicated in the upregulated (+) and downregulated (−) gene sets. The overall response includes all genes which responded at each pH, and the common response represents the transcripts which were up- or downregulated at both pH 3 and 5. Values with both + and − indicate significant enrichment of functional categories in both up- and downregulated clusters. The significance of each category is numerically indicated as a $\log_{10} P$ value (as described in Materials and Methods [2]).

TABLE 4. Overrepresentation of transcription factor binding sites among lactic acid-responsive genes[a]

Transcription factor(s)	Significance ($\log_{10} P$ value)				
	Overall		Common	Specific	
	pH 3	pH 5		pH 3	pH 5
Ace2p				(+) 3.89	
Aft2p		(+) 6.17			(+) 4.39
		(−) 3.15			(−) 5.05
Cin5p		(+) 4.43			(+) 4.02
		(−) 6.94			(−) 5.51
Gcn4p		(−) 3.03			(−) 3.18
Gln3p		(−) 3.36			
Hap1p	(+) 6.77	(+) 3.30	(+) 4.66		
Nrg1p	(+) 3.76	(+) 5.94			(+) 4.12
Phd1p	(+) 3.04	(+) 4.71			(+) 3.22
Rcs1p/Aft1p		(+) 11.82	(+) 3.20		(+) 8.90
Skn7p		(−) 4.25			
Sko1p		(+) 8.66			(+) 7.69
Sok2p		(+) 3.54			(+) 3.02
Ste12p		(−) 3.52			(−) 3.61
Stp1p/Bap1p	(−) 3.10		(−) 3.97		
Sut1p		(+) 4.24			
Swi5p	(+) 3.82			(+) 4.68	
Tec1p		(−) 5.22			(−) 4.47
Yap1p	(−) 3.24			(−) 4.14	
Yap7p	(−) 3.70			(−) 4.84	

[a] Overrepresentation of transcription factor binding sites among lactic acid-responsive genes identified in a comparison of lactate-challenged and reference anaerobic chemostat cultures of *S. cerevisiae* CEN.PK 113-7D at pH 3 and pH 5. Overrepresentation of binding sites for each transcription factor is indicated in the upregulated (+) and downregulated (−) gene sets. Enrichment of transcription factor binding among the common genes and the pH-specific gene sets are also indicated. The significance of each category is numerically indicated as a $\log_{10} P$ value (as described in Materials and Methods [2]).

tassium cations. The overrepresentation of the MIPS categories "ionic and cationic homeostasis" in this gene set (Table 4) further supports this interpretation.

Binding of several transcription factors involved in cell morphology was overrepresented among genes that showed a lactate response (of either up- or downregulation) at pH 5 only. However, the regulation patterns observed gave no clear indication for either transcriptional induction or repression of pseudohyphal growth. For example, the upregulated gene set at pH 5 shows enrichment of genes that are bound by both negative (Nrg1p, Sok2p) and positive (Phd1p) regulators of pseudohyphal growth. Furthermore, two additional transcription factors involved in cell morphology (Ste12p, Tec1p) were overrepresented among genes that were specifically downregulated at pH 5. Given the elevated concentrations of salts in these cultures, osmotic responses mediated by the Sho1 receptor may contribute to enhanced signaling of pseudohyphal growth-related transcriptional responses (41).

The involvement of genes pertaining to the cell cycle and pseudohyphal growth was also apparent at pH 3, but the identity of the transcription factors for which binding was overrepresented was different than that at pH 5. For instance, targets of Ace2p, which controls the cell cycle by activating the expression of early G_1-specific genes, were enriched at pH 3. In addition to the role of Ace2p in cell cycle control, Gancedo (17) suggested that Ace2p may also influence pseudohyphal growth. Furthermore, targets of Swi5p, a transcription factor that activates transcription of genes expressed in G_1 phase and at the G_1/M boundary, were overrepresented at low pH. Although extensive transcriptional events related to cellular mor-

TABLE 5. Changes in expression of genes involved in iron homeostasis in lactate-challenged chemostat cultures compared to reference anaerobic chemostat cultures[a]

Gene(s)	Description[b]	Fold change at:	
		pH 3	pH 5
AFT1/RCS1	Transcription factor involved in iron utilization and homeostasis; activates the expression of target genes in response to changes in iron availability	1.0	3.3
AFT2	Iron-regulated transcriptional activator, required for iron homeostasis and resistance to oxidative stress	1.0	1.6
ARN1	Transporters that specifically recognize siderophore-iron chelates	2.3	34
ARN3/SIT1	Transporters that specifically recognize siderophore-iron chelates; transcription is induced during iron deprivation and diauxic shift	1.0	11
ARN4/ENB1	Endosomal ferric enterobactin transporter, expressed under conditions of iron deprivation; member of the major facilitator superfamily; expression is regulated by Rcs1p	1.8	4
ATX1	Cytosolic copper metallochaperone that transport copper to the secretory vesicle copper transporter Ccc2p for eventual insertion into Fet3p	1.9	4
CCC2	Cu^{2+}-transporting P-type ATPase, required for export of copper from the cytosol into an extracytosolic compartment	2.0	7
CTH1	Member of the CCCH zinc finger family; may function with Tis11p in iron homeostasis	1.0	2.1
CTR1	High-affinity copper transporter of the plasma membrane; mediates nearly all copper uptake under low-copper conditions; transcriptionally induced at low-copper levels and degraded at high-copper levels	−2.2	−2.7
FET3	Ferro-O_2-oxidoreductase required for high-affinity iron uptake and involved in mediating resistance to copper ion toxicity; belongs to the class of integral membrane multicopper oxidases	7.4	103
FET4	Low-affinity Fe(II) transporter of the plasma membrane	1.3	−20
FIT1	Mannoprotein that is incorporated into the cell wall via a glycosylphosphatidylinositol (GPI) anchor; involved in the retention of siderophore-iron in the cell wall	1.5	8
FIT2	Mannoprotein that is incorporated into the cell wall via a GPI anchor; involved in the retention of siderophore-iron in the cell wall	1.9	60
FIT3	Mannoprotein that is incorporated into the cell wall via a GPI anchor; involved in the retention of siderophore-iron in the cell wall	3.1	57
FRE1	Ferric reductase and cupric reductase; reduces siderophore-bound iron and oxidized copper prior to uptake by transporters; expression induced by low copper and iron levels	−1.5	1.3
FRE2	Ferric reductase and cupric reductase; reduces siderophore-bound iron and oxidized copper prior to uptake by transporters; expression induced by low copper and iron levels	1.5	32
FRE3	Ferric reductase, reduces siderophore-bound iron prior to uptake by transporters; expression induced by low iron levels	1.0	9
FRE5	Putative ferric reductase with similarity to Fre2p; expression induced by low iron levels	1.0	3
FRE6	Putative ferric reductase with similarity to Fre2p; expression induced by low iron levels	1.0	3
FTR1	High affinity iron permease involved in the transport of iron across the plasma membrane; forms complex with Fet3p; expression is regulated by iron	2.2	1.7
HMX1	Endoplasmic reticulum-localized, heme-binding peroxidase involved in the degradation of heme; does not exhibit heme oxygenase activity despite similarity to heme oxygenases; expression regulated by Aft1p	1.5	6
ISU1	Conserved protein of the mitochondrial matrix; performs a scaffolding function during assembly of iron-sulfur clusters; interacts physically and functionally with yeast frataxin (Yfh1p)	1.0	2.1
ISU2	Conserved protein of the mitochondrial matrix, required for synthesis of mitochondrial and cytosolic iron-sulfur proteins, performs a scaffolding function in mitochondria during Fe/S cluster assembly	2.1	2.3
MRS4	Mitochondrial iron transporter of the mitochondrial carrier family (MCF), very similar to and functionally redundant with Mrs3p; functions under low-iron conditions; may transport other cations in addition to iron	1.0	2.4
SMF3	Putative divalent metal ion transporter involved in iron homeostasis; transcriptionally regulated by metal ions; member of the Nramp family of metal transport proteins	1.6	2.2
TIS11	mRNA-binding protein expressed during iron starvation; binds to a sequence element in the 3′ untranslated regions of specific mRNAs to mediate their degradation; involved in iron homeostasis	4.1	45
YBR047W	Mitochondrial protein of unknown function; contains Rcs1p and Aft2p binding domains (58)	1.0	19
YHL035C	Putative vacuolar multidrug resistance protein; contains Rcs1p and Aft2p binding domains (58)	1.0	27

[a] Relative transcript levels of genes involved in iron homeostasis in lactate-challenged and reference anaerobic chemostat cultures of *S. cerevisiae* CEN.PK 113-7D at pH 3 and pH 5. Fold change is relative to that of reference anaerobic chemostat cultures (with no lactic acid added) at the corresponding pH.
[b] Gene descriptions originate from the *Saccharomyces* Genome Database (www.yeastgenome.org) and from the references noted.

phology and cell cycle were observed, routine phase-contrast microscopy of the chemostat cultures did not reveal clear morphological differences.

High concentrations of lactate anions cause an "iron status" transcriptional response. At pH 5, the majority of the transcriptional response to lactate focused on metal ion homeostasis. In particular, many genes related to the cellular iron status often showed a very strong upregulation in lactate-challenged cultures (Table 5). Essentially, this transcriptional response to lactate is strikingly similar to the response that would be observed for iron-limited cultures (28, 43). Although copper and iron homeostasis are linked (55), both copper-independent and copper-dependent mechanisms were upregulated in the lactate-challenged cultures, indicating that the changes observed were not a secondary effect of copper status. This "iron status response" observed for lactate is not observed for four other organic acids tested under similar conditions (2). Furthermore, this response was almost completely alleviated at pH 3, where the total acid concentration was lower (Table 5) and the concentration of anionic species was drastically lower (840 mM at pH 5; 60 mM at pH 3).

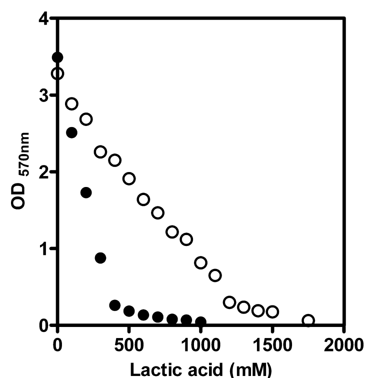

FIG. 2. Relative free-iron concentration, as indicated by an optical density at 570 nm (OD_{570nm}) in complete synthetic medium supplemented with increasing concentrations of lactic acid at pH 5 (●) and pH 3 (○). For each condition, 150 μM $FeSO_4$ was added, and unbound Fe^{2+} was detected with ferrozine (250 μM), which results in absorbance at 570 nm upon interaction with Fe^{2+} (47). Decreased OD_{570nm} indicates decreased availability of Fe^{2+} in growth medium containing increasing concentrations of the lactate anion. However, the quantitative relationship between free Fe^{2+} and OD_{570nm} is unknown.

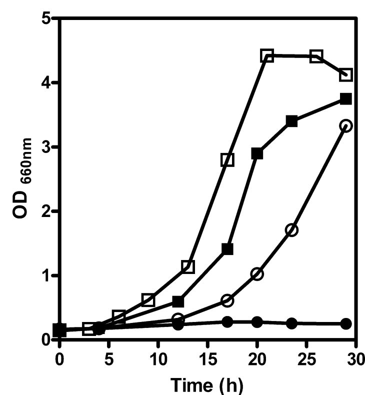

FIG. 3. Growth of the *S. cerevisiae* mutant strain (*aft1*Δ) in glucose synthetic medium containing 900 mM lactic acid. Iron sulfate concentrations in the synthetic medium were 10 μM (standard concentration, ●), 100 μM (○), and 250 μM (■). In contrast to the *aft1*Δ strain, the reference strain (CEN.PK 113-7D) did not exhibit a growth deficiency in the presence of lactic acid with the standard concentration of iron (□). The pH of all shake flasks was set to 5, and urea was utilized as the nitrogen source to prevent acidification of the growth medium (21).

It is well known that lactate chelates iron (and, to a lesser degree, other metal cations) in a pH-dependent manner (54). To confirm the decreased availability of iron in the culture medium in the presence of lactic acid, a simple ferrozine assay was performed. Ferrozine binds with free Fe^{2+} to produce a purple color (47). Although approximately 10 μM $FeSO_4$ was added to the growth medium, the presence of EDTA in the medium (required to keep metal cations in solution) likely binds the majority of iron. Consequently, the free-iron levels in the growth medium were not detectable with the ferrozine assay. However, upon supplementation of all growth media with additional $FeSO_4$ (150 μM), a signal could be detected. Growth media containing a range of lactic acid concentrations (at each pH) were supplemented with iron and analyzed spectrophotometrically. The addition of 250 μM ferrozine (Sigma) revealed a clear relationship between lactic acid concentration and "free" iron (Fig. 2). Furthermore, pH dependency was demonstrated as the decrease in iron was much more severe at pH 5 than at pH 3.

To investigate the physiological relevance of the iron homeostasis response to lactate, *AFT1*, which encodes a key transcriptional regulator, was deleted. The deletion mutant failed to grow in shake-flask cultures (pH 5) on standard synthetic medium with glucose when 900 mM lactate was added. Supplementation of the synthetic medium with 10-fold or 25-fold higher $FeSO_4$ concentrations partially rescued this lactate-induced growth deficiency (Fig. 3), while growth of the reference strain was unaffected by iron supplementation (data not shown).

Within the categories that are more highly enriched at, or specific to, pH 5, there are many genes coding for enzymes that require iron and/or other divalent cations for activity (based on the BRENDA database [www.brenda-enzymes.info/], using the enzyme classification code). Many enzymes did not have data available for *S. cerevisiae*, but divalent metal cations were extensively required in other organisms. In reference to amino acid metabolism, 2 of the 12 genes (~17%) downregulated in the common response encode enzymes which require divalent metal cations, while 14 of the 18 enzymes (~78%) which were downregulated only at pH 5 showed the same requirement. Similar trends were observed for metabolism of energy reserves and C compound and carbohydrate metabolism, where 92% and 75% of the genes downregulated at pH 5, respectively, indicated a requirement for metal cations.

Haa1p regulon: transcriptional analysis and phenotypic screening. The transcription factor Haa1p is not included in a published compendium of transcription factor-binding data (20) and was therefore not included in the statistical analysis of the sets of lactate-responsive genes. However, previous studies of yeast responses to poorly lipophilic organic acids (16) suggest that Haa1p may be involved in the tolerance of lactic acid. The overrepresentation of amine/polyamine transport among the lactate-upregulated genes is also indicative of the importance of this regulon, as the known Haa1p targets *TPO2* and *TPO3* are involved in polyamine and organic acid transport. Indeed, 6 of the 10 genes which have previously been shown to be regulated by Haa1p were significantly upregulated in response to lactic acid at pH 3 (Table 6). Even though the concentration of total acid is almost twofold higher at pH 5, the role of Haa1p appears to be more pronounced at pH 3, strongly suggesting that this regulon responds primarily to undissociated lactic acid. Although the transcription factor itself was not strongly induced by lactic acid, a number of target genes were highly upregulated. The transporters of the major facilitator superfamily (*TPO2* and *TPO3*) along with *YGP1* (a poorly characterized cell wall glycoprotein implicated in other stress responses [13, 32]) and *YRO2* (homologous to *HSP30*) were highly upregulated in the presence of lactic acid. Similarly, the importance of *TPO2*, *TPO3*, *YGP1*, and, to a lesser extent, *YRO2*, was also highlighted in response to other organic acids with low to moderate membrane affinities (16).

Given the strong transcriptional induction of this regulon, a *haa1*Δ mutant and a set of isogenic strains that carried deletions in the most highly upregulated Haa1p targets were

TABLE 6. Transcriptional modulation of previously identified target genes of the Haa1p transcriptional regulator[a]

Gene	Transcript fold change at:	
	pH 3	pH 5
HAA1	1.2	1.3
TPO2	39.5	8.2
TPO3	4.2	2.4
YGP1	8.0	1.9
PHM8	1.1	−1.1
YRO2	8.2	1.2
GRE1	1.0	1.0
YIR035C	2.1	3.7
YLR297W	1.0	1.7
YPR157W	3.2	1.9
YER130C	−1.1	2.1

[a] Relative transcript levels of previously identified target genes of the Haa1p transcriptional regulator in lactate-challenged and reference anaerobic chemostat cultures of *S. cerevisiae* CEN.PK 113-7D at pH 3 and pH 5. Fold change is relative to that of reference anaerobic chemostat cultures (with no lactic acid added) at the corresponding pH.

TABLE 7. Growth rates of *S. cerevisiae* CEN.PK 113-7D and mutant strains[a]

Genotype	Avg specific growth rate (h^{-1})	
	500 mM lactic acid	750 mM lactic acid
Reference	0.25	0.19
*haa1*Δ	0.16	No growth[b]
*tpo2*Δ	0.22	0.17
*tpo2*Δ *tpo3*Δ	0.25	0.17
*yro2*Δ	0.24	0.19
*ygp1*Δ	0.23	0.20

[a] Specific growth rates of *S. cerevisiae* CEN.PK 113-7D (reference strain) and those of strains carrying deletions in the *HAA1* gene or in the genes belonging to the Haa1p regulon that were most strongly upregulated in response to lactic acid (Table 6). Each strain was grown at pH 3 in anaerobic batch cultures in the presence of the indicated concentration of lactic acid. Specific growth rates (h^{-1}) are averages of two independent experiments for each concentration of lactic acid. Data for replicate growth experiments differed by less than 15%.

[b] No change in CO_2 concentrations were measured in the off gas after nearly 200 h.

screened for sensitivity to lactic acid at pH 3. Duplicate anaerobic batch cultures of the mutant strains were compared to the reference strain at pH 3 (Table 7). A significant growth defect was observed for *haa1*Δ in the presence of 500 mM lactic acid. Furthermore, the *haa1*Δ strain had not shown any growth after almost 200 h in the presence of 750 mM lactic acid, while the reference strain grew at 0.19 h^{-1}. Consistent with these data, a previous study with *haa1*Δ showed a prolonged lag phase in the presence of acetic acid, and the duration of the lag phase was directly correlated to increased intracellular accumulation of the acid (16). Strangely, the deletion of *TPO2* (the most highly upregulated gene of the regulon) did not result in a lactate-induced growth defect. We hypothesized that *TPO3*, which is 89% identical to *TPO2* (30), was compensating for the lack of *TPO2*. However, a *tpo2*Δ *tpo3*Δ double mutant did not exhibit a growth defect in the presence of high lactic acid concentrations (Table 7). Similarly, the deletion of *YGP1* or *YRO2* had no effect on growth rates in the presence of lactic acid.

DISCUSSION

Is lactic acid an atypical weak-organic-acid preservative? By analyzing physiological responses to lactic acid (pK_a = 3.86) at pH 3 and at pH 5, we attempted to dissect the effects of the lactate anion (the predominant species at pH 5) from those of the undissociated lactic acid (predominant at pH 3). It is well documented that the antimicrobial actions of weak-organic-acid preservatives such as sorbate, benzoate, and propionate are conferred primarily by extracellular undissociated acid. This species causes dissipation of the pH gradient across the plasma membrane and intracellular accumulation of the anion (25, 31, 48, 51). Defense mechanisms against this mode of toxicity include increased activity of the plasma membrane ATPase and ATP-driven export of organic acids (23, 24, 42, 45). Indeed, the effects of benzoate on biomass yields at pH 3 could be accurately predicted from experimental data obtained at pH 5 by (i) assuming that the undissociated acid is the sole extracellular species responsible for toxicity and (ii) applying the Henderson-Hasselbach equation for benzoic acid dissociation.

The observation that biomass formation in anaerobic chemostat cultures was not directly correlated to the concentration of undissociated lactic acid in the cultures (Fig. 1) suggested that undissociated lactic acid was not the only species affecting the biomass yield on glucose. This clear difference between lactate and other weak organic acids is probably related to the low lipid solubility of lactic acid (the octanol-water partition coefficient [log P = −0.60]). Lipid solubility is strongly correlated with weak-organic-acid toxicity. For example, reducing the biomass yield of *S. cerevisiae* to 50% of that of the reference condition required 105 mM of acetate (log P = −0.31), 2 mM of benzoate (log P = 1.87), 20 mM of propionate (log P = 0.33), or 1.3 mM of sorbate (log P = 1.33) at pH 5 (2), while under the same conditions, 900 mM of lactate was required to achieve the same effect (Fig. 1; this study). At these high concentrations of the lactate anion, a substantial fraction of the consumed glucose was redirected toward glycerol formation as an osmoregulation response (22) to the high osmotic strength of the growth medium used for the experiments at pH 5. While, at first glance, osmotic response might seem to be a trivial consequence of the experimental design, it represents a realistic concern for the high-level industrial production of lactate at pH values above that of the pK_a of 3.86, where lactic acid formation has to be titrated with hydroxide or carbonate salts.

Iron homeostasis: involvement of the Aft1p regulon in lactate tolerance. The strong and coordinated induction of a large number of target genes of the Rcs1p/Aft2p and Aft1p transcription factors, as well as the heme-responsive regulator Hap1, indicated a strong impact of lactate on the regulation of iron homeostasis. The physiological functions of these genes indicated a general remodeling of iron metabolism, including uptake, retention, and incorporation (28). Transcriptional effects of lactate on iron homeostasis genes were also recently reported for shake-flask cultures of *S. cerevisiae* (29). The present chemostat study demonstrated that, while this effect was very pronounced at pH 5, it was largely alleviated at pH 3. Ferrozine assays provided further support for the hypothesis that this iron homeostasis response was caused by chelation of free iron at high concentrations of lactate anions, thus severely

restricting its bioavailability, and probably not, as previously proposed, by an increased iron requirement of lactate-stressed yeast cells (29).

Experiments with the *aft1*Δ strain indicated that the Aft1p regulon, involved in iron homeostasis, is essential for lactate tolerance in *S. cerevisiae.* The clear lactate sensitivity of the *aft1*Δ mutant, which was not found in a previous study (29), could be complemented by iron supplementation of the medium. The absence of a measurable effect of iron supplementation on the specific growth rate of lactate-stressed cultures of a wild-type reference strain indicates that the observed transcriptional reprogramming of iron homeostasis genes is sufficient to counter the effects of iron chelation by lactate. In view of these results, especially at low pH, iron chelation is unlikely to represent a major issue for industrial implementation of industrial processes for the production of lactate with engineered *S. cerevisiae* strains.

Transcriptional responses to undissociated lactic acid: involvement of the Haa1p regulon in lactic acid tolerance. The majority of the genes known to be regulated by the Haa1p regulon, which has previously been implicated in tolerance to other weak organic acids (16), were strongly upregulated in the presence of lactate. This effect was most pronounced at pH 3, consistent with the notion that the Haa1p regulon is involved in tolerance to the undissociated acid.

The identification of lactate export mechanisms is highly relevant because energy costs for export have been implicated in the inabilities of engineered "homolactic" *S. cerevisiae* strains to gain a net ATP yield from lactate fermentation (60). Of the 10 genes hitherto identified as Haa1p targets, *TPO2* and *TPO3* encode H^+ antiporters (49) belonging to the major facilitator superfamily. Originally, *TPO2* and *TPO3* were thought to be polyamine transporters localized to the vacuole (57); however, there is evidence for plasma membrane localization (4), and deletion of *TPO2* has been correlated to increased accumulation of intracellular acetate (16). Involvement of Tpo2p and/or Tpo3p in lactate/proton antiporter activity, combined with proton expulsion via the plasma membrane ATPase, which has an ATP/proton stoichiometry of 1 (7, 53), would be consistent with a zero net ATP yield for lactate fermentation (60). However, since even a double deletion of *TPO2* and *TPO3* did not result in impaired growth in the presence of lactic acid, either the encoded transporters are not involved in lactate tolerance or redundant exporters are encoded by the *S. cerevisiae* genome.

The strong growth defect of the *haa1*Δ strain in the presence of lactic acid (Table 7) was not found for single-deletion strains in the transcriptionally upregulated targets of Haa1p. This indicates either that the proteins encoded by the Haa1p regulon have to act synergistically to achieve lactate tolerance or, alternatively, that other as-yet-unknown targets of the Haa1p regulon are involved in lactate tolerance. A thorough investigation of the composition of the Haa1p regulon and its mechanistic contributions to lactate tolerance is therefore warranted.

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