

Copper and Iron Are the Limiting Factors for Growth of the Yeast *Saccharomyces cerevisiae* in an Alkaline Environment*

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Exposure of the yeast *Saccharomyces cerevisiae* to an alkaline environment represents a stress situation that negatively affects growth and results in an adaptive transcriptional response. We screened a collection of 4825 haploid deletion mutants for their ability to grow at mild alkaline pH, and we identified 118 genes, involved in numerous cellular functions, whose absence results in reduced growth. The list includes several key genes in copper and iron homeostasis, such as *CCC2*, *RCS1*, *FET3*, *LYS7*, and *CTR1*. In contrast, a screen of high-copy number plasmid libraries for clones able to increase tolerance to alkaline pH revealed only two genes: *FET4* (encoding a low affinity transporter for copper, iron, and zinc) and *CTR1* (encoding a high affinity copper transporter). The beneficial effect of overexpression of *CTR1* requires a functional high affinity iron transport system, as it was abolished by deletion of *FET3*, a component of the high affinity transport system, or *CCC2*, which is required for assembly of the transport system. The growth-promoting effect of *FET4* was not modified in these mutants. These results suggest that the observed tolerance to alkaline pH is because of improved iron uptake and indicate that both iron and copper are limiting factors for growth under alkaline pH conditions. Addition to the medium of micromolar concentrations of copper or iron ions drastically improved growth at high pH. Supplementation with iron improved somewhat the tolerance of a *fet3* strain but was ineffective in a *ctr1* mutant, suggesting the existence of additional copper-requiring functions important for tolerance to an alkaline environment.

Extracellular pH represents one of the most important factors influencing cell physiology and growth. The yeast *Saccharomyces cerevisiae* grows better at acidic pH than in neutral or alkaline media, and maintenance of an acidic environment is mainly based on the activity of the plasma membrane H⁺-ATPase, which actively extrudes protons. Maintenance of a

proton gradient is crucial for the uptake of diverse nutrients and cations (1, 2) and, consequently, even a transient exposure to mild alkaline pH represents a stress situation for *S. cerevisiae* to which the yeast must adapt to survive and proliferate (3).

Adaptation of *S. cerevisiae* to alkaline pH involves a change in its expression profile. DNA microarray analysis identified more than 300 genes that are induced at least 2-fold when cells are transferred from acidic medium to one of pH 7.6–8.0 (4–6). This transcriptional response occurs through different transduction mechanisms including the calcineurin pathway (6), the Rim101/Ngr1 pathway (5, 7), and others yet to be identified. Different studies identified a common set of induced genes, which include a substantial number of genes relevant for the metabolism of copper and iron cations, such as *FRE1*, *FET3*, *CTR1*, *ARN1*, *ARN3*, and others (4–6). We utilized two complementary genetic screens to define the mechanisms behind adaptation to an alkaline environment: a search in a systematic deletion mutant library for genes whose absence would increase sensitivity to alkaline pH, and a screen for genes that, in high-copy number, could increase the tolerance to high pH of wild-type yeast. The results of these approaches point to the acquisition of iron and copper as the limiting factors for the growth of yeast cells under such conditions.

MATERIALS AND METHODS

Bacterial and Yeast Strains and Growth Conditions—*Escherichia coli* DH5 α was used as a host for DNA cloning experiments. Bacterial cells were grown at 37 °C in LB medium containing, when needed, 50 μ g/ml ampicillin for plasmid selection. *S. cerevisiae* cells were grown at 28 °C in YPD medium (10 g/liter yeast extract, 20 g/liter peptone, and 20 g/liter dextrose) or, when indicated, in synthetic minimal or complete minimal medium (8). The strains used in this work derive from DBY746 (MAT α *ura3-52 leu2-3,112 his3- Δ 1 trp1- Δ 239*) or BY4741 (*Mat a his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0*).

Recombinant DNA Techniques—*E. coli* cells were transformed by standard treatment with calcium chloride (9). *S. cerevisiae* cells were transformed by a modification of the lithium acetate method (10). Restriction mapping, DNA ligations, and other recombinant DNA techniques were carried out by standard methods (9).

Screening of the Systematic Deletion Library for Alkaline pH Sensitivity—A systematic *kanMX* deletion library constructed in the BY4741 genetic background (11) was grown in YPD medium supplemented with 150 μ g/ml G418 up to saturation (3–4 days). The cultures were replicated using a stainless steel 96-pin replicator (Nalge Nunc Intl.) at a density of 384 clones/plate on Omnigrid plates (Nunc) containing YPD-agar supplemented with 50 mM TAPS¹ and adjusted at pH 6.2 (control plates), 7.2, and 7.5. Growth was recorded after 24 and 48 h and evaluated visually by two different individuals. Clones that showed weak or no macroscopic growth after 48 h at any of the alkaline pHs tested were considered as positives. These clones were recovered from the original 96-well plates, diluted with YPD up to an A₆₆₀ of 0.05, and

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¹ The abbreviations used are: TAPS, 3-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]-1-propanesulfonic acid; ORF, open reading frame; VPS, vacuolar protein sorting; Sod, superoxide dismutase.

TABLE I
Mutations that result in growth defect under alkaline conditions.

Numbers in parentheses indicate the level of alkaline sensitivity (1 being the highest sensitivity). Genes in bold type correspond to those identified in a recent report (11) as conferring a fitness defect when deleted. For details, see the main text.

Function	Alkaline pH sensitive mutants
Vacuole organization and biogenesis	<i>CUP5</i> (1), <i>TFP1</i> (1), <i>VMA2</i> (1), <i>VMA22</i> (1), <i>VMA4</i> (1), <i>VMA5</i> (1), <i>VMA6</i> (1), <i>VMA7</i> (1), <i>CWH36</i> (1), <i>VMA21</i> (2), <i>PEP3</i> (2), <i>PEP5</i> (2), <i>PPA1</i> (2), <i>TFP3</i> (2), <i>VMA13</i> (2), <i>VPS16</i> (2), <i>VPS33</i> (2), <i>VPS34</i> (2), <i>ARP5</i> (3), <i>VPS15</i> (3), <i>FAB1</i> (4), <i>PEP7</i> (4), <i>VMA8</i> (4), <i>VPS65</i> (5)
Transport in other organelles	<i>GCSI</i> (3), <i>SEC22</i> (3), <i>DRS2</i> (3), <i>VPS20</i> (4), <i>GLO3</i> (4), <i>COG8</i> (4), <i>SYS1</i> (4)
Metal ion homeostasis	<i>CTR1</i> (1), <i>RCS1</i> (1), <i>SOD1</i> (1), <i>LYS7</i> (2), <i>SOD2</i> (2), <i>CCC2</i> (4), <i>FET3</i> (4), <i>PMR1</i> (4)
Metabolism	
Aminoacid metabolism	<i>GLY1</i> (1), <i>PHO2</i> (1), <i>PRO1</i> (1), <i>CYS3</i> (3), <i>ARO2</i> (4), <i>ILV1</i> (4), <i>TYR1</i> (5)
Lipid metabolism	<i>ERG2</i> (2), <i>ERG6</i> (2), <i>ARV1</i> (3), <i>BTS1</i> (3), <i>AGP2</i> (4), <i>DAP1</i> (4), <i>FEN1</i> (4), <i>MGA2</i> (4)
Phosphate metabolism	<i>PHO4</i> (2), <i>PHO81</i> (2), <i>PHO85</i> (3)
Protein biosynthesis	<i>TEF4</i> (3), <i>FES1</i> (4), <i>HCR1</i> (4), <i>RPL13B</i> (4), <i>RPL27A</i> (4)
Cell polarity, cell wall organization and biogenesis	<i>RHO4</i> (1), <i>BUD25</i> (1), <i>BEM1</i> (2), <i>GAS1</i> (2), <i>SLT2</i> (2), <i>BCK1</i> (3), <i>BEM4</i> (4), <i>CNB1</i> (5)
Chromatin modification, architecture & transcription	<i>SWI3</i> (1), <i>BUR2</i> (3), <i>DAL81</i> (3), <i>GCN5</i> (3), <i>HTZ1</i> (3), <i>REF2</i> (3), <i>REG1</i> (3), <i>RPB9</i> (3), <i>SPT20</i> (3), <i>SSN8</i> (3), <i>UME6</i> (3), <i>ADA2</i> (4), <i>KEM1</i> (4), <i>MED2</i> (4), <i>SPC72</i> (4), <i>SRB5</i> (4), <i>SWI4</i> (4), <i>TOP1</i> (4), <i>MRS1</i> (5)
Others	<i>PHO86</i> (1), <i>KEX2</i> (1), <i>RIB4</i> (1), <i>DIA2</i> (2), <i>SPS1</i> (2), <i>FYV6</i> (3), <i>MAP1</i> (3), <i>TPD3</i> (3), <i>ADE12</i> (4), <i>ATP1</i> (4), <i>ATP11</i> (4), <i>NEM1</i> (4), <i>PTC1</i> (4), <i>URE2</i> (4), <i>ZWF1</i> (4), <i>COX16</i> (5), <i>RIB1</i> (5), <i>SCP160</i> (5)
Unknown	<i>YEL045C</i> (1), <i>YKL118W</i> (1), <i>YOR331C</i> (1), <i>YJL175W</i> (3), <i>HUR1</i> (4), <i>ILM1</i> (4), <i>PKR1</i> (4), <i>YLR358C</i> (4), <i>YJR018W</i> (5), <i>YOR251C</i> (5), <i>YOR305W</i> (5)

evaluated in duplicate for pH sensitivity by drop test on YPD plates ranging from pH 6.8 to 7.5 (in steps of 0.1–0.2 pH units). The intensity of the phenotype was scored from 1 (the most sensitive) to 5, on the basis of the lowest pH at which the strain showed no or marginal growth after 48 h. In several cases, the mutation conferring sensitivity to alkali was generated in a different wild-type background to confirm the phenotype.

Screening for Genes Able to Confer in High-copy Number Tolerance to Alkaline pH—The wild-type strain DBY746 was transformed with two different genomic libraries constructed in the multicopy plasmids YEp24 (a generous gift of Dr. Ramón Serrano, Universidad Politécnica de Valencia) and YEp13 (American Type Culture Collection 37415), respectively. About 48,000 transformants were recovered in plates containing synthetic medium lacking uracil (YEp24) and 100,000 in plates lacking leucine (YEp13). Clones from each library were recovered in 1 ml of selective medium and plated at a density of 4000 clones/plate in YPD plates containing 50 mM TAPS and adjusted with potassium hydroxide at different pHs (10 plates/pH condition) in the range 7.3–7.8. Clones able to form macroscopic colonies after 3 days in the plates at pH 7.6–7.8, in which cells carrying an empty plasmid were unable to grow, were considered as positive.

The gene *FET4* was isolated from the genomic clone B6 by digestion with HindIII and XhoI and ligation of the resulting 3.4 kbp fragment (which contains the entire open reading frame (ORF) flanked by 1117 bp of 5' region and 610 bp of 3' region) into the HindIII and Sall sites of plasmid YEplac195 (12), to produce plasmid pFET4. The same strategy with clone 2.1.2 produced pFET4-s, which contains only 442 bp of 5' promoter sequence. The gene *CTR1* was recovered from clone B15 as a 2.7-kbp EcoRV fragment containing the entire ORF flanked by 1040 bp 5' and 378 bp 3' regions, respectively, and cloned into the SmaI site of YEplac195.

Other Techniques—Growth in plates and liquid medium at high pH was monitored essentially as described previously (6). All pH values were determined after autoclaving the medium, and pH of the plates was measured using a surface electrode. When metal ions were added, cells were resuspended at an A_{620} of 0.002 in liquid YPD medium buffered at the appropriate pH and supplemented from a freshly prepared 25-fold concentrated solution of the desired salt: $(\text{NH}_4)_2\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$ for iron; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ for copper; and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ for zinc. Addition of the salts did not affect the pH of the medium.

RESULTS

Screening of a Systematic Deletion Mutant Library for Mutations that Confer Sensitivity to Alkaline Conditions—To identify mutations that would confer a growth defect under alkaline conditions, a library of 4825 haploid deletion mutants was arrayed at a density of 384 clones/plate on YPD plates buffered at pH 6.2 (standard growth conditions), 7.2, and 7.5 (alkaline conditions) and visually screened for formation of macroscopic

colonies after 48 h. More than 300 clones that showed reduced or no growth under alkaline conditions were subjected to a more detailed test by growing dilutions of the cultures at a range of pHs (6.2, 6.8, 7.2, 7.3, and 7.5). The final output of the screen was the identification of 118 genes whose deletion confers sensitivity to alkaline pH. The degree of sensitivity varied greatly, and it was quantified, according to the lowest pH able to severely impair growth, from 1 (the highest sensitivity) to 5 (only slightly more sensitive than the isogenic wild-type strain). We found genes involved in a variety of cellular processes, in many cases not previously related to pH tolerance (Table I), suggesting that many different aspects of the yeast biology could be compromised and become limiting for growth in an alkaline environment. Although a detailed analysis of all of them is beyond the scope of this work, it is worth noting that we were able to identify in our screening a large number of genes (24) involved in vacuolar organization and biogenesis, including most of the components of the hydrogen-exporting vacuolar ATPase complex. A significant number of mutations in genes relevant for sterol metabolisms, such as *ERG2*, *ERG6*, and *ARV1*, and response to phosphate starvation (*PHO2*, *PHO81*, *PHO85*) were also identified. Further examination of a set of mutants defective in phosphate response indicated that the *pho4* mutant and, particularly, a *pho84 pho89* strain, lacking high affinity phosphate uptake, were also sensitive to high pH (not shown). In addition, our screen detected quite a few mutants for genes related to different aspects of iron and copper homeostasis: *FET3* and *CTR1*, responsible for the high affinity uptake of copper and iron, respectively; *LYS7* and *CCC2*, which are intracellular copper transporters; *RCS1/AFT1*, a transcription factor responsible for induction of a set of genes under iron starvation; or *SOD1*, a copper and zinc superoxide dismutase. This led us to evaluate the relative impact of alkaline pH in the growth of a set of mutants related to copper and iron metabolism. As can be observed in Fig. 1, *ctr1*, *rcs1*, *sod1*, and *lys7* account for the most dramatic effect among the mutants tested. We also observed a slight growth defect in *ptr1* and *fet4* mutants, which was unnoticed under the conditions of the original screening.

***FET4* and *CTR1* Are the Only Genes Whose Overexpression Is Able to Improve Growth under Alkaline pH**—The result of the screen revealed that alteration of many different functions could impair the ability of yeast cells to adapt to alkaline

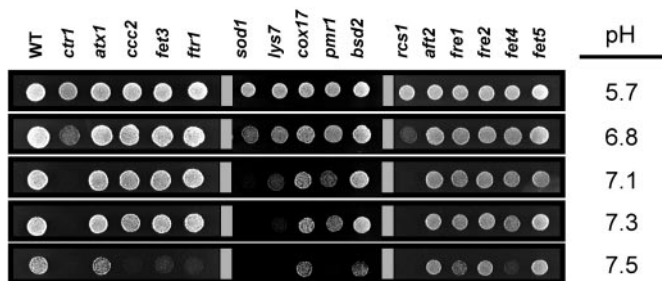


FIG. 1. Sensitivity to alkaline pH of strains carrying deletions of diverse genes involved in copper and iron homeostasis. The wild-type strain BY4741 and the indicated isogenic mutants were grown in YPD-50 mM TAPS plates adjusted to the indicated pH. Growth was monitored after 48 h at 28 °C.

conditions. To elucidate which of those functions could represent a limiting factor for growth at alkaline pH, we identified genes that, when overexpressed, would confer to wild-type yeast strain the ability to growth at high pH. A first screen was performed by plating about 40,000 clones transformed with a genomic library in the high-copy number YEp24 vector. 71 positive clones were obtained; 30 of those clones were retested for pH tolerance, and then the plasmid was subsequently recovered. Restriction mapping of the inserts revealed that they corresponded only to two different genomic regions that appeared roughly at 50% frequency and are represented by clones B6 and B15 (Fig. 2). A second screen was performed using a different genomic library. An equivalent number of clones were plated, and a total number of 46 putative positives were recovered. Upon analysis of the plasmids, two different genomic regions, represented in Fig. 2 as clones 2.1.2 and 5.3.4, were identified. Comparison of all four clones clearly showed that clones B6 and 2.1.2 define overlapping genomic regions, having in common the *FET4* ORF, whereas clones B15 and 5.3.4 share the *CTR1* ORF. This evidence suggested that the *CTR1* and *FET4* genes were responsible for the alkaline tolerance phenotype. Subcloning of the appropriate regions in the YEp195 plasmid allowed confirmation of this hypothesis (Fig. 2). Simultaneous high-copy number expression of both genes did not result in increased tolerance to alkaline pH (not shown). As observed, *FET4* was somewhat less effective than *CTR1* in increasing pH tolerance. However, a version of *FET4* (pFET-s) that lacks five of the six sequences required for inhibition by the Rox1 repressor (13, 14) was as effective as *CTR1* (not shown). Therefore, the observed difference could be the result of a different level of expression.

The Tolerance Conferred by Overexpression of *CTR1* Requires a Functional *Fet3* Protein—As mentioned above, *CTR1* encodes a high-affinity (K_m 1–4 μM) plasma membrane copper transporter (15, 16), whereas *FET4* codes for a low affinity (K_m around 35 μM), broad specificity plasma membrane transporter that is able to mediate uptake of iron, copper, and other metal cations (17–21). Therefore, uptake of copper seems to be the common characteristic for both gene products. However, it is known that high affinity uptake of iron, mediated by the Fet3 oxidase-Ftr1 permease complex, requires an efficient uptake of copper (16), thus raising the possibility that the tolerance conferred by overexpression of *CTR1* could be because of a secondary effect resulting from improved uptake of iron. To test this possibility, we overexpressed *CTR1* and *FET4* in strains lacking *FET3*, as well as in *ccc2* mutants devoid of the protein that delivers copper to Fet3 (22, 23), and in *atx1* mutants, which lack a metallochaperone that provides copper to Ccc2 (24, 25). Fig. 3 shows a schematic depiction of the functional relationship between these proteins. Overexpression of *FET4* was able to improve growth at alkaline pH in all of these mutants with

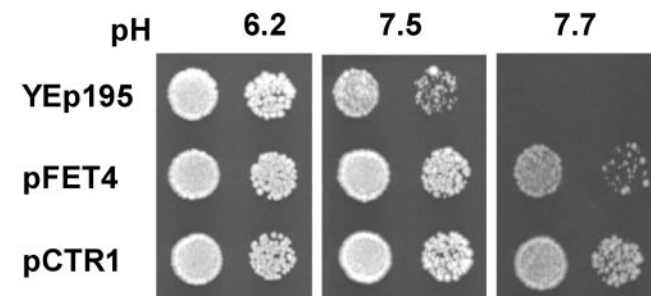
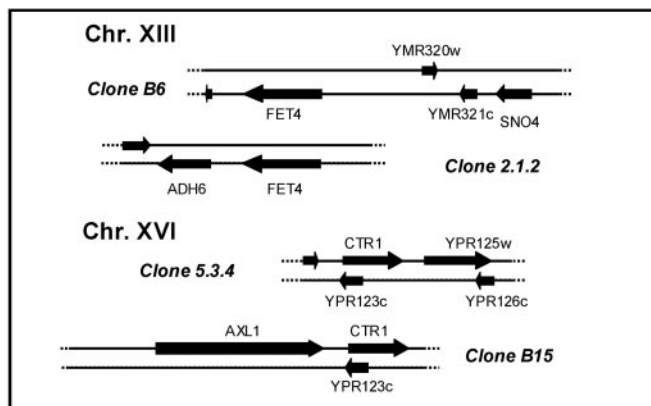


FIG. 2. Identification of *FET4* and *CTR1* as genes able to increase in high-copy number tolerance to alkaline pH. *Upper panel*, structure of the genomic regions isolated in the screening described in the main text (denoted as continuous lines). Clones B15 and B6 were obtained from the genomic library constructed in YEp24, whereas clones 2.1.2 and 5.3.4 derive from the YEp13 library. *Lower panel*, the *FET4* and *CTR1* genes were isolated from genomic clones and introduced in high-copy number into the wild-type BY4741 strain. Transformants (pFET4 and pCTR1, respectively) were grown in selective medium and their tolerance was tested on YPD-50 mM TAPS plates at the indicated pH. Growth was monitored after 72 h at 28 °C.

the same efficiency as in a wild-type strain (Fig. 3). In contrast, expression of *CTR1* in cells lacking Fet3 or Ccc2 did not increase tolerance. This result suggests that the beneficial effect of *CTR1* is mainly because of an improved iron uptake. Expression of the copper transporter was able to increase somewhat the tolerance of an *atx1* strain. This observation is consistent with the existence of an Atx1-independent way to furnish Ccc2 with copper (24, 25).

The finding that expression of the metal transporters Fet4 and Ctr1 were able to improve growth at alkaline pH suggests that the availability of copper and/or iron may be limiting factors for the growth of yeast cells in an alkaline ambient. This is consistent with the increased expression of genes responsible for uptake and utilization of these metals when cells are grown in an alkaline ambient (4–6).

Supplementation of the Medium with Copper or Iron Ions Increases Tolerance to Alkaline pH—To test the possibility that lack of copper and/or iron may be responsible for failure to grow at high pH, we carried out a set of experiments in which the medium was supplemented with these transition metals. Addition of copper in the low micromolar range (1–10 μM) was able to dramatically improve the growth of a wild-type strain at alkaline pH (Fig. 4). Supplementation of media with iron had a similar effect, although higher concentrations were needed (5–50 μM). Addition of both copper and iron had a synergistic effect as, at a concentration of 5 μM copper, the presence of very low concentrations of iron were sufficient to allow maximal growth at the pH tested. Addition of zinc cations, also transported by *FET4*, did not confer tolerance at all of the concentrations tested (1–100 μM).

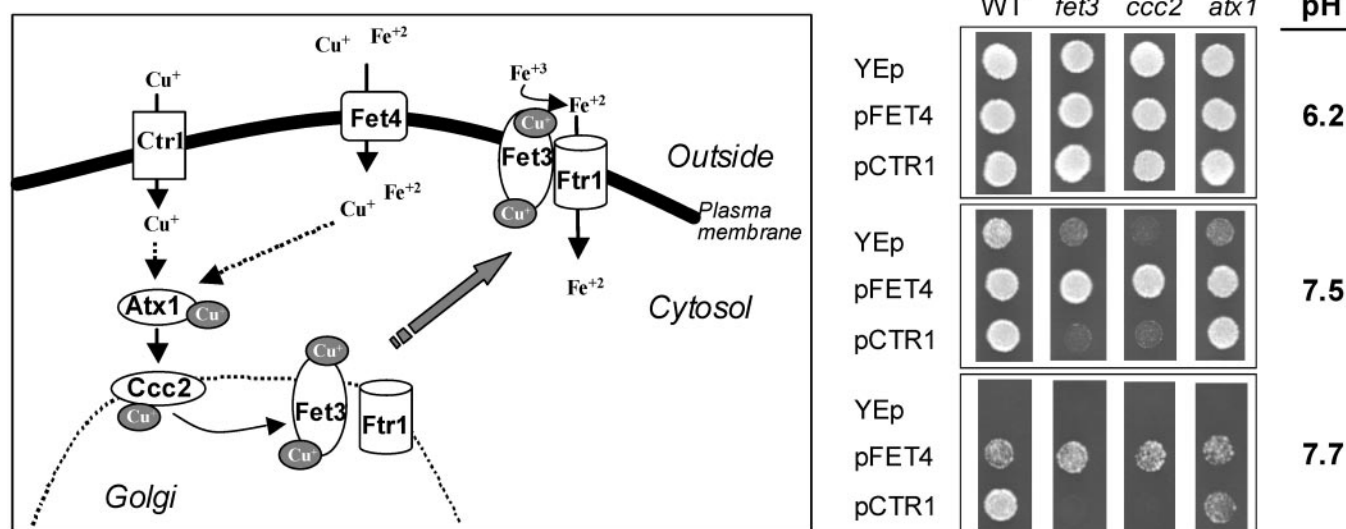


FIG. 3. Tolerance to high pH conferred by overexpression of *CTR1* requires an intact *Fet3* function. The *FET4* and *CTR1* genes were introduced in high-copy number into the wild-type BY4741 strain as well as in isogenic strains lacking *FET3*, *CCC2*, or *ATX1* genes, and their tolerance to different pHs was tested as in Fig. 2. The relationship between these genes and their relevance in the metabolism of copper and iron is depicted schematically at the left.

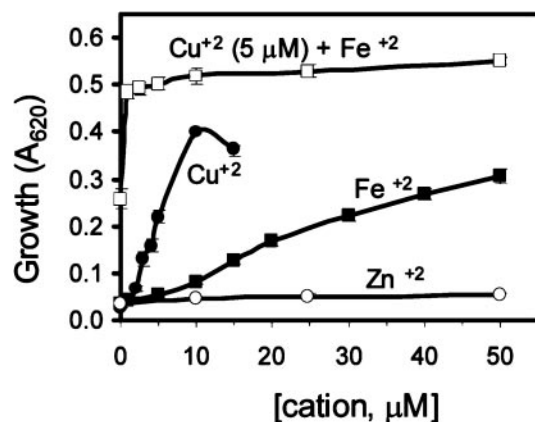


FIG. 4. Effect on the tolerance to alkaline pH of supplementation of the medium with iron, copper, or zinc cations. Cultures of the wild-type strain DBY746 were inoculated as described under "Materials and Methods" in YPD-50 mM TAPS adjusted to pH 8.0 in the absence or the presence of the indicated concentrations of metal cations. Growth was monitored by determining the A_{620} of the cultures after 20 h at 28 °C.

To gain insight into the role of copper and iron ions under alkaline conditions, we tested the effect of supplementation of these cations in strains lacking specific components of their uptake and transport pathways. The addition of copper improved the growth of an *atx1* mutant but was completely ineffective in *fet3* or *ccc2* cells (Fig. 5, upper panel). These results support the view that increased copper improves growth at alkaline pH by increasing uptake of iron. Supplementation with iron (Fig. 5, lower panel) significantly improved the growth of the *atx1* strain and, when added at high concentrations (75–100 μM), allowed growth of *fet3* and *ccc2* cells with equal efficiency. Addition of copper at relatively low concentrations was sufficient to sustain growth of a *fet4*-null mutant but, as expected, did not allow growth of a *ctr1* strain (Fig. 5, lower panel). Although iron was sufficient to promote growth of the *fet3* strain (although relatively high concentrations were needed), the *ctr1* mutant did not grow even at the higher concentration of iron tested. This suggests that copper is required at alkaline pH not only to mediate an efficient iron uptake, but also to fulfill other physiological roles.

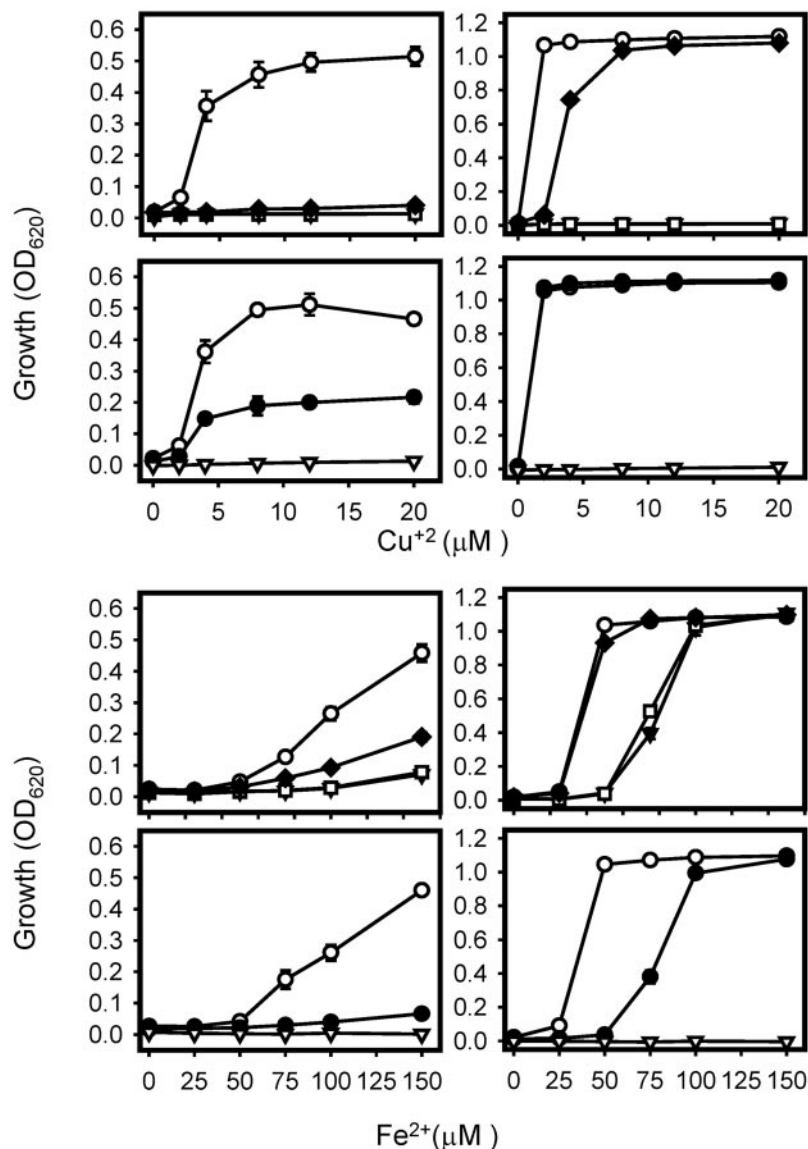
DISCUSSION

Growth at alkaline pH requires a number of physiological adaptations. We present evidence that the availability of copper and iron is a key factor limiting growth of baker's yeast in an alkaline environment. A genome-wide analysis identified a rather large number of genes required for growth at alkaline pH, pointing to a variety of functions as possibly limiting factors for growth at high pH. For instance, mutation of several genes related to response to phosphate starvation resulted in sensitivity to alkali. As exposure to high pH results in a dramatic increase in the transcriptional response of genes required for efficient uptake and metabolism of phosphate (4–6), it was reasonable to consider the lack of available phosphate as a possible limiting factor for growth.

Among the mutations conferring a severe sensitivity to alkali, we found genes required for the efficient uptake of iron and copper. Iron and copper are transition metals involved in redox reactions that are essential for all eukaryotes, but whose intracellular concentrations must be carefully monitored, as they are potentially toxic (26). High affinity copper uptake is mediated by the products of the *CTR1* and *CTR3* genes (15, 16, 27, 28). Low affinity copper uptake can be mediated by a number of membrane transporters, including *Fet4*, which transports with similar affinity iron, as well as zinc, cadmium, and other cations (17–20), and *Smf1*, a member of the *Nramp* metal transporter family, which also shows a broad metal specificity (29). Because there is virtually no free copper available in the cytosol (30), copper-requiring enzymes rely on the existence of intracellular transporters, called metallochaperones, which deliver copper to the different intracellular compartments where this metal can be incorporated to the target enzymes.

Uptake of iron by *S. cerevisiae* can be performed through four pathways: high affinity uptake, mediated by an iron permease, *Smf1*, *Fet4*, and siderophore uptake (for a recent review, see Ref. 31). High affinity iron uptake occurs through a complex formed by the high affinity iron permease *Ftr1* and the multi-copper oxidase *Fet3* (22, 32–34). The substrate for both iron and copper transport is the lower of the two valence states of the elemental metal, which requires the activity of the cell surface reductases, the products of the *FRE1* and *FRE2* genes. The entire system is transcriptionally regulated by the *Rcs1/*

FIG. 5. Effect of iron and copper supplementation in the tolerance to alkaline pH of mutants lacking relevant genes for copper and iron homeostasis. The wild-type strain BY4741 (○) and the isogenic *fet3* (▼), *ccc2* (□), *atx1* (◆), *fet4* (●), and *ctr1* (▽) mutants were tested for growth in liquid medium at pH 8.1 in the presence of diverse concentrations of copper (upper panel) or iron (lower panel) cations, as indicated previously. Growth was monitored after 24 (left) or 40 h (right), as indicated. Data correspond to a representative experiment and are presented as the mean \pm S.D. from cultures in triplicate.



Aft1 or Aft2 transcription factors (35–38). The high affinity iron uptake requires an efficient copper uptake, because maturation of Fet3 in the secretory apparatus involves acquisition of copper delivered by Ccc2 and the copper chaperone Atx1 (22). The absence of genes required for intracellular copper transport leading to maturation of Fet3, such as *CTR1*, *ATX1*, or *CCC2*, results in a deficiency in Fet3 activity and a decrease in high affinity iron transport (15, 16, 22, 24, 39).

The requirement for expression of the iron and copper regulators for growth at alkaline pH, and the effect of increased expression of *CTR1* or *FET4*, can be accounted for by the chemistry of iron and copper. Both metals show a reduced solubility at alkaline pH. The lowered availability can be overcome by either increasing the concentration of the metal (Fig. 4) or by increasing the expression of the transporters. In this regard, we have observed that the induction of Ctr1 expression observed after alkaline stress (6)² is not observed in cells lacking the Mac1 copper-sensing transcription factor (data not shown), indicating that cells do sense an intracellular copper starvation under alkaline conditions. In agreement with this notion, we have tested the sensitivity to alkali of a *mac1* deletion

(initially not present in our mutant collection) and found that it is highly sensitive to alkali (not shown). It may seem odd that, given the different existing mechanisms for copper and/or iron uptake in *S. cerevisiae*, only two of such genes could improve growth when overexpressed. However, it must be noted that overexpression of siderophore uptake genes, such as *ARN1-4*, would probably be ineffective, as *S. cerevisiae* does not produce this type of compound (40, 41). On the other hand, the relatively high affinity Smf1 iron and copper transporter is believed to act as a metal/ H^+ cotransporter and, therefore, its activity is probably marginal in cells facing an alkaline environment (29, 42).

The decreased ability of many of the deletion strains to grow in alkaline pH can be accounted for, at least in part, by the decreased activity of high affinity iron transport system. Not only do deletions in the structural components of the transport system affect growth, but deletions in genes required to assemble the system also affect growth. Thus, copper loading of apoFet3 requires an acidic environment in the vesicular apparatus (22, 43). Therefore, defects in acidification, through defects in the vacuolar H^+ -ATPase, will lead to defective assembly of the high affinity iron transport system and thus an inability to grow at alkaline pH. Similarly, the effect of several apparently unrelated mutations can be explained through its

² L. Viladevall, R. Serrano, G. Doménech, J. Giraldo, A. Barceló, and J. Ariño, manuscript in preparation.

repercussion on vacuolar function. Thus, mutations in the ergosterol biosynthetic pathway, such as *erg2*, *erg6*, or *arv1*, could affect endocytosis and vacuolar function (44, 45). Mutation of *ARV1* and *FEN1* are known to affect sphingolipid metabolism (45, 46). Interestingly, sphingolipids have been recently shown to be required for generation of a functional V_1 component of the vacuolar ATPase (46). A very recent genome-wide search for genes required for survival under iron-deprivation conditions (47) has revealed an unsuspected connection between *CHW36* (identified in our study as a mutation highly sensitive to alkalization), iron deprivation, and vacuolar acidification. From our work, it also becomes clear that alteration of membrane trafficking has a dramatic effect on alkaline tolerance, as indicated by the severe alkaline phenotype of the class C VPS (vacuolar protein sorting) encoding genes *VPS11* (*PEP5*), *VPS16*, *VPS18* (*PEP3*), and *VPS33*, components of the homotypic fusion and protein sorting complex involved in docking of vesicles with the target membranes (see Ref. 48 and references therein). However, it should be noted that mutations in structural components of the vacuolar ATPase or in genes related to vacuolar function have a much more dramatic effect on alkaline tolerance than those produced by the absence of Fet3 or Ccc2 (see Table I). In addition, we have observed that many of these mutants do not improve alkaline tolerance in the presence of added copper or iron (not shown). Therefore, it is reasonable to assume that the effect of certain vacuolar mutations is not solely due to a deficient copper loading of apoFet3.

We observed that supplementation of the medium with copper or iron was unable to improve growth of not only a *ctr1* strain (Fig. 5) but also that of a *lys7* mutant (data not shown). *Lys7* (also known as *Ccs1*) is the copper chaperone that delivers and inserts copper into the Cu/Zn superoxide dismutase (*Sod1*) (49), a widely distributed enzyme required to handle reactive oxygen species. Mutations in the *SOD1* gene are responsible for a number of human diseases (for reviews, see Refs. 50 and 51). Yeast cells lacking *Sod1* are highly sensitive to oxygen and to agents that lead to oxidative stress, such as paraquat or menadione (52). Our observation that mutations or deletions in *SOD1*, *SOD2*, and *LYS7* result in similar sensitivity to alkaline pH suggests the possibility that alkaline stress may also lead to oxidative stress and that a certain supply of copper would be necessary to face this situation. This possibility is currently under investigation in our laboratory, and would explain our observation that, whereas the tolerance conferred to alkali by overexpression of *Ctr1* is dependent upon Fet3, the alkaline phenotype of a *fet3* mutant is not as dramatic as that of a *Ctr1*-deficient strain.

While this work was in progress, a report appeared describing the parallel analysis of a nearly complete *S. cerevisiae* collection of tagged gene mutants by hybridization to high-density oligonucleotide arrays under a variety of conditions, including exposure to pH 8.0 (11). These authors identified 128 alkali-hypersensitive mutants, a number similar to the one reported here. However, comparison of both sets of data reveals little overlap (20 genes, shown in *boldface* in Table I). Among the mutants found in common, we observed all levels of sensitivity, as determined in our assay. To extend the comparison, we selected 41 mutants showing the highest fitness defect according to Ref. 11 and tested them for growth on YPD plates at a range of alkaline pHs (not shown). Only eight of those mutants showed a detectable growth defect (five of them were already identified in our screen), whereas the other three (*YMR073c*, *YMR099c*, and *KRE1*) showed a very slight growth defect at the highest pH tested. Most probably the different methodology and conditions used in both approaches may reasonably account for the differences in the final output. How-

ever, we wish to stress that the approach followed by Giaever *et al.* (53) failed to identify mutants in components of the vacuolar H^+ -ATPase, which are known to be sensitive to alkali. In contrast, our screening was able to identify mutants in virtually all subunits of this ATPase.

Our studies demonstrate that changes in pH can lead to metabolic adaptations to transition metal availability. It is known that the supply of iron can represent a limitation for growth, virulence, and/or invasiveness of pathogens, such as *Candida albicans* (54–56). For this pathogen, environmental pH serves as a signal for morphological differentiation, neutral or alkaline conditions favoring the switch from yeast to hyphal growth form, which has been postulated to be essential for virulence (for a recent review, see Ref. 57). It may well be that the developmental switch reflects a response to a nutritional deficiency.

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