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

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Raquel Serrano‡§, Dolores Bernal§, Ernesto Simón¶, and Joaquín Arino||
From the Departament de Bioquímica i Biologia Molecular, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain

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 DH5α was used as a host for DNA cloning experiments. Bacterial cells were grown at 37 °C in LB medium containing, when needed, 50 μg/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 (MATα ura3–52 leu2–3,112 his3–11,15 trp1–239) or BY4741 (Mat a his3Δ1 leu2Δ0 met15Δ0 ura3Δ0).

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 μg/ml G418 up to saturation (3–4 days). The cultures were replicated using a stainless steel 96-pin replicator (Nalge Nunc Int.) at a density of 384 clones/plate on Omnigrid plates (Nunc) containing YPD agar supplemented with 50 μg/ml TAPS† 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 A600 of 0.05, and

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‡ Recipient of a fellowship from the Ministerio de Ciencia y Tecnología, Spain.
§ Both authors contributed equally to this work.
¶ Recipient of a fellowship from the Universitat Autònoma de Barcelona.
|| To whom correspondence should be addressed: Dept. Bioquímica i Biologia Molecular, Facultat de Veterinària, Ed. V, Universitat Autònoma de Barcelona, Bellaterra, 08193 Barcelona, Spain. Tel.: 34-93-5812182; Fax: 34-93-5812006; E-mail: Joaquin.Arino@uab.es.

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

Mutations that result in growth defect under alkaline conditions.

| Function                                      | Alkaline pH sensitive mutants                                                                 |
|----------------------------------------------|---------------------------------------------------------------------------------------------|
| Vacuole organization and biogenesis         | CUPS1 (1), TPPI (1), VMA2 (4), VMA22 (1), VMA4 (1), VMA5 (1), VMA6 (1), VMA7 (1), CWH36 (1), VMA21 (2), PEPI (2), PEPS2 (2), PPA1 (2), TPP3 (2), VMA13 (2), VPS16 (2), VPS33 (2), VPS34 (2), ARPS5 (3), VPS15 (3), FAB1 (4), FEP7 (4), VMA8 (4), VPS65 (5) |
| Transport in other organelles                | GCS1 (3), SEC22 (3), DRSS2 (3), VPS20 (4), GLO3 (4), COG8 (4), SYS1 (4)                   |
| Metal ion homeostasis                        | CTR1 (1), RCS1 (1), SOD1 (1), LYS7 (2), SOD2 (2), CCC2 (4), FET3 (4), PMRI (4)            |
| Metabolism                                   | GLY1 (1), PHO2 (1), PRO1 (1), CYS3 (3), ARO2 (4), IVL1 (4), TYR1 (5)                      |
| Aminoacid metabolism                         | ERG2 (2), ERG6 (2), ARV1 (3), BTS1 (3), AGP2 (4), DAPI (1), FEN1 (4), MGA2 (4)           |
| Lipid metabolism                             | PEFO4 (2), PHO81 (2), PHO65 (3)                                                           |
| Phosphate metabolism                         | TEF3 (3), FET3 (4), HCR1 (4), RPL3B (4), RPL27A (4)                                      |
| Protein biosynthesis                          | RHQ4 (1), BUD53 (1), BEM1 (2), GAS1 (2), S1L2 (2), BCK1 (3), REM4 (4), CNN4 (5)          |
| Cell polarity, cell wall organization and biogenesis | SWI3 (1), BUR2 (3), DALA1 (3), GCNS (3), HZT27 (3), REF2 (3), REG1 (3), RBP9 (3), SPT20 (3), SSNS8 (3), UME6 (3), ADA2 (4), KEM1 (4), MED2 (4), SSCP2 (4), SRB5 (4), SWI4 (1), TOP1 (4), MR51 (5) |
| Chromatin modification, architecture & transcription | PHO86 (4), KEKX2 (1), BIRA (2), DIA2 (2), SPS1 (2), FTY6 (2), MAP1 (3), TPZ3 (3), ADE12 (4), ATPI (4), ATP11 (4), RMI1 (4), FTC1 (4), URE2 (4), ZWFI (4), COX16 (5), RIB1 (5), SCB160 (5) |
| Others                                       | YEL604C (1), YKL118W (1), YOR331C (1), YJL175W (3), HURL (4), ILMI (4), PKRI (4), YLR358C (4), YJR101W (5), YOR251C (5), YOR365W (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. Ramon Serrano, Universidad Politecnica 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 ml of selective medium and plated at a density of 4000 clones/plate in YPD 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 kb 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 SacI sites of plasmid YEpplac195 (12), to produce plasmid pFET4. The same strategy with clone 2.1.2 produced pFET4-s, which contains only 442 bp of 5’ region and 610 bp of 3’ regions, respectively, and cloned into the SmaI site of YEpplac195.

**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 A590 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: (NH4)2Fe(SO4)2·4H2O for iron; CuSO4·5H2O for copper; and ZnSO4·7H2O 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, 7.5, and 7.8). The final output of the screen was the identification of 118 genes whose deletion conferred 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 ftr1 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
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To improve growth at alkaline pH in all of these mutants with 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 a 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).
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Materials and Methods

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 A₆₂₀ 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 ctrl strain (Fig. 5, lower panel). Although iron was sufficient to promote growth of the fet3 strain (although relatively high concentrations were needed), the ctrl 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 possible limiting factors for growth at high pH. For instance, mutation of several genes related to response to phosphate starvation resulted in sensitivity to alkalai. 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 Pfr1 and the multicopper 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/
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 \textit{CTR1}, \textit{ATX1}, or \textit{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 \textit{CTR1} or \textit{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 Ctrl1 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 alkaline 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 \textit{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 \textit{ARN1}–4, would probably be ineffective, as \textit{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\textsuperscript{+} 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 the vesicular \textit{H\textsuperscript{+}-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

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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 V1 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 (Sod)1 (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 we would explain our observation that, whereas the tolerance conferred to alkaliphiles 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. However, 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 alkaliphiles. 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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