Multicopy suppression screening of *Saccharomyces cerevisiae* identifies the ubiquitination machinery as a main target for improving growth at low temperature

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A decrease in ambient temperature alters membrane functionality and impairs the proper interaction between the cell and its external milieu. Understanding how the cells adapt membrane properties and modulate the activity of membrane-associated proteins is therefore of major interest from both the basic and applied point of view. Here, we have isolated multicopy suppressors of the cold sensitivity phenotype of a trp1 strain of *Saccharomyces cerevisiae*. Three poorly characterized genes, the endocytic adaptor *ALY2*, the J protein *CAJ1*, and the ubiquitin C-terminal hydrolase *UBP13*, were identified as mediating increased growth at 12°C of both Trp⁻ and Trp⁺ yeast strains. This effect was likely due to down-regulation of cold-instigated degradation of nutrient permeases since it was missing in mutant cells of the ubiquitin ligase *RSP5*. Indeed, we found that 12°C-treatments reduced the level of several membrane transporters, including Tat1p and Tat2p, two yeast tryptophan transporters, and Gap1, the general amino acid permease. We also found that the lack of Rsp5p increased the steady state level of Tat1p and Tat2p, and that *ALY2*-engineered cells grown at 12°C had higher Tat2p and Gap1p abundance. Nevertheless, high copy number of *ALY2* or *UBP13* improved cold-growth even in the absence of Tat2p. Consistent with this, *ALY2*- and *UBP13*-engineered cells of the industrial QA23 strain grew faster and produced more CO₂ at 12°C as compared with the parental when maltose was used as sole carbon source. Hence, the multicopy suppressors isolated in this work appear to contribute to the correct control of the cell surface protein repertoire and their engineering might have potential biotechnological applications.
A major requirement for strain improvement involves stress tolerance and adaptability of cells to environmental stressors in industrial applications (22, 47). For example, some industrial processes involving industrial strains of *Saccharomyces cerevisiae*, like brewing and some wine fermentations, take place at temperatures around 10-12°C, which is far below the optimal temperature for this organism (~28°C). Growth at low temperature reduces the production by *S. cerevisiae* of higher alcohols and increases the amount of esters (10, 20). The low fermentation temperature also has a prominent effect on primary flavours which are retained to a greater degree. However, under these conditions, an extended lag phase before the onset of vigorous fermentation activity is observed, which reduces the cost-effectiveness and efficiency of production. In wine making, this lag phase also increases the risk of halted or sluggish fermentation (20). Hence, cold tolerance is an important biotechnological trait and there is an urgent need for strains able to ferment at low temperatures both quickly and in a reproducible way.

Like other stressors, cold influences the structural and functional properties of cellular components negatively, both physically and chemically (4). Cold modifies enzyme kinetics (28, 58), and increases the molecular order of membrane lipids, *i.e.*, rigidification (33), affecting the membrane-environment and thus the activity of membrane-associated enzymes and transporters. Key processes such as plasma membrane ATPase activity (53), the higher proton motive force (39), and the transport of various amino acids (60) depend thus on temperature-instigated changes in membrane fluidity and become limiting factors for cell growth.

In support of this view, previous reports have shown that tryptophan uptake is impaired after a downward shift in temperature (1) and that several cold-sensitive mutants are affected in tryptophan transport and biosynthesis (3, 12, 52). It has also
been suggested that the sensitivity of tryptophan permeases to changes in membrane fluidity could determine or influence the growth temperature profile of tryptophan auxotroph strains of *S. cerevisiae* (1, 2). Bearing this in mind, we hypothesized that enhanced membrane fluidity might rescue cold-mediated growth inhibition of Trp- yeast strains (48). Indeed, production in *S. cerevisiae* of sunflower desaturases (*FAD2* genes) increased the content of dienoic fatty acids and fluidity of the yeast membrane; however, growth was diminished in the recombinant *FAD2* strains at low temperatures (48). Thus, membrane fluidity appears to be essential in determining cold tolerance in *S. cerevisiae*, although its exact effect on amino acid uptake and growth is unclear.

The impact on yeast physiology of cold stress also depends on how low temperature affects the composition of membrane proteins. It has been reported that cold triggers the degradation of the tryptophan transporter Tat2p via the ubiquitination pathway (2, 42). Ubiquitination is a reversible post-translational modification of cellular proteins, playing a central role in the regulation of protein degradation and trafficking (31, 41). In particular, Rsp5p-dependent ubiquitination of Tat2p has been observed in starved yeast cells (8). The yeast ubiquitin ligase Rsp5p (a homolog of mammalian Nedd4 family proteins) controls most trafficking-related ubiquitination events at the plasma membrane and at other membranes (9). In agreement with this, overproduction of Tat2p, or mutation of *DOA4, UBP6,* or *UBP14*, encoding ubiquitin-specific proteases, confers increased cold-growth to Trp- yeast cells (1, 38). Altogether, these results suggest that Rsp5p may play a role in the degradation of Tat2p in response to low temperature, although no direct evidence of this function has been reported. Likewise, the putative adaptor proteins involved in this regulatory process are unknown, nor is it known whether cold may trigger the down-regulation of other membrane proteins.
Here, we have performed a multicopy suppressor analysis of the cold sensitivity phenotype of a trp1 mutant strain of *S. cerevisiae*. Our hypothesis was that this screening could reveal, among others, genes governing plasma membrane properties. However, all the genes identified encode proteins directly or indirectly involved in ubiquitination machinery. We have exploited these effects to identify previously uncharacterized actors and genetic interactions that appear to be important in remodeling the membrane-protein repertoire at low temperature. Finally, we demonstrate that engineering the ubiquitination machinery can potentially reverse growth inhibition associated with cold stress in industrial strains.

**MATERIALS AND METHODS**

**Strains, culture media and general methods.** The laboratory strains used in this study are described in Table 1. A haploid derivative (*ho*) of the diploid wine yeast strain QA23 (Lallemand, Montreal, Canada) was kindly provided by J. M. Guillamón. Auxotrophic mutants of the QA23 ho strain, MJHL201 (Ura−) and MJHL213 (Ura− Trp−) were constructed as described below. Yeast cells were cultured at 30 or 12°C in YPD media (1% yeast extract, 2% peptone and 2% glucose) or SCD (0.67% yeast nitrogen base without amino acids, DIFCO, plus 2% glucose) supplemented with the appropriate amino acid drop out (ForMedium, England). Yeast transformants carrying the geneticin (kanMX4), nourseothricin (natMX4) and hygromycin (hphMX4) resistant module were selected on YPD agar plates containing 200 mg/l of G-418 (Sigma), 50 mg/l of nourseothricin (clonNAT, WERNER Bioagents, Germany) or 300 mg/l of hygromycin B (ForMedium), respectively (25, 61). Ura− derivatives were selected by plating an
unmutagenized population of cells on SCD-agar supplemented with uracil (10 mg/l), proline (1 g/l) and 5-fluoroorotic acid, 5-FOA (1 g/l) (37). Auxotrophs for tryptophan were recovered as 5-fluoroanthranilic acid (FAA)-resistant mutants. FAA-containing SCD medium was prepared by the addition of 0.5 mg/ml FAA (10% w/v in ethanol) as previously described (59). *Escherichia coli* DH5α host strain was grown in Luria-Bertani (LB) medium (1% peptone, 0.5% yeast extract and 0.5% NaCl) supplemented with ampicillin (50 mg/l). All amino acids, sugars and antibiotics were filter-sterilized and added to autoclaved medium. Solid media contained 2% agar. Yeast cells were transformed by the lithium acetate method (29). *E. coli* was transformed by electroporation following the manufacturer's instructions (Eppendorf). A stock solution of 25 mM phytosphingosine (PHS) was prepared in ethanol, sampled in small volumes and stored at -20°C until use. For plate phenotype experiments cultures were diluted to OD$_{600}$ = 0.8 and 10-fold serial dilutions spotted (3 μl) onto SCD- or YPD-agar solid media. In some experiments, maltose was used instead of glucose as sole carbon source (SCM medium). Unless otherwise indicated, colony growth was inspected after 2-4 days of incubation at 30°C. Cold-growth experiments were carried out at 12°C for 8-12 days.

For galactose induction of GFP-tagged genes, cells were pre-grown overnight in SCD, and then refreshed (OD$_{600}$ ~ 0.25) in SC containing galactose as sole carbon source (SCG). After 90 min, 2% glucose was added and the culture was transferred to a 12°C-chilled water bath for the times indicated.

**Screening of multicopy suppressors, DNA manipulations and sequencing.** Cold multicopy suppressors were isolated from a *S. cerevisiae* genomic library constructed in the vector YEp24 (*URA3/2 µm*; kindly provided by B.A. Morgan). Transformants were selected on YPD medium containing 25 μM PHS at 12°C. Plasmid DNA was rescued,
amplified in *E. coli* and tested according to standard manipulations (51). The insert of each plasmid conferring cold-tolerance was sequenced using YEp24-forward and -reverse primers (Supplementary Table S1). DNA sequencing was performed by the dideoxy-chain termination procedure (50). Analysis of sequence data was carried out using DNAMAN sequence analysis software (Lynnon Biosoft). Similarity searches were performed at the National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) using BLAST software (5). Search of conserved domains was carried out by scanning the sequences against Conserved Domain Databases (CDD) at NCBI (35). Alternatively, protein domains were searched against the PROSITE database of protein families (54) at the Swiss Institute of Bioinformatics, SIB (http://www.isb-sib.ch). Sequence alignment was done using the ALIGN software at the GENESTREAM network server (http://xylian.igh.cnrs.fr/bin/align-guess.cgi).

**Plasmid and disruption strain construction.** The YCplac33- (*URA3; 24*) based plasmids p3HA-TAT1c and p3HA-TAT2c, encoding N-terminally hemagglutinin (HA)-tagged Tat1 or Tat2 proteins, respectively (2), were a gift from Fumiyoshi Abe. The plasmid YCpJ25, containing a *GAP1* fusion with *GFP* under the control of the *pGAL1* (18) was provided by Sébastien Léon. PCR-amplified fragments containing the whole sequence of *ALY2, EXO70, TRL1, YJL086C, CAJ1, ISD11, TPA1, AST1, PRS4, KTI11* and *UBP13* gene, including its own promoter and terminator, were obtained with specific synthetic oligonucleotides (Table S1) and genomic DNA as template. Amplification was carried out under standard conditions. The corresponding fragments were digested with the appropriate set of enzymes, KpnI/PstI (*ALY2*), SalI/XbaI (*EXO70, ISD11, PRS4*), EcoRI/XbaI (*TRL1, YJL086C, KTI11*), EcoRI/PstI (*CAJ1*), SalI/PstI (*TPA1*), EcoR1/SacI (*AST1*) or XbaI/PstI (*UBP13*), and cloned into the plasmid YEplac195 (*URA3; 24*) digested with the same set of enzymes. The marker
swap plasmid pUL9 (14) cut with SmaI was used in some cases in order to change the URA3 marker to LEU2. Disruption-deletion cassettes for ALY2, TAT1 and TAT2 were prepared by PCR using specific synthetic oligonucleotides (Table S1) and plasmids pFa6A (kanMX4), pAG25 (natMX4) and pAG28 (hphMX4) as template, respectively (25, 61). Correct gene disruption was detected by diagnostic PCR (27), using a set of oligonucleotides (Table S1), designed to bind outside of the replaced gene sequence and within the marker module (data not shown).

Preparation of protein extracts and Western blot analysis. For crude extracts, 10 OD600 units of cells were harvested by centrifugation at 1,090 x g and resuspended in 300 µl of lysis TNE buffer (50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH = 7.5) supplemented with a protease inhibitor cocktail (Roche, cat# 1861278). Then, glass beads (acid-washed, 0.4 mm) were added, and the mixture was vortexed three times for 1 min, with 1 min on ice between each mixing. Cells debris and unbroken cells were removed by centrifugation at 500 x g for 5 min, and the supernatant was incubated with SDS-loading buffer (Fermentas, cat# R0891) at 95ºC for 10 min. Finally, 10 µl of each sample was separated by SDS–PAGE, blotted onto nitrocellulose membranes, and filters were blocked with 5% milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween-20).

Gap1-GFP was detected by using a monoclonal anti-GFP antibody (Roche Diagnostics, clones 7.1 and 13.1). HA-Tat1p and HA-Tat2p were visualized with an anti-HA rabbit polyclonal antibody (Santa Cruz Biotechnology, cat# sc-805). A specific anti-Hxk2p serum raised in rabbits by immunization with a purified fraction of hexokinase PII (46) was used as loading control of total protein. The antisera were applied at 1:3,000 dilutions. As secondary antibody, we used horseradish peroxidase-conjugated goat anti-rabbit (1:2000, cat# 7074, Cell Signaling, Danvers, MA) or rabbit
anti-mouse (1:5,000, Dako, cat# P0260). Blots were developed using the ECL Western blotting detection kit by Pierce (Rockford, IL) or the ECL Advance system by GE Healthcare (Waukesha, WI). Images were captured with the Las-1000 Plus imaging system (Fuji, Kyoto, Japan).

**Fermentative performance.** The fermentation capacity of the yeast strains under study was evaluated by measuring CO₂ production. Yeast biomass from QA23 transformants was prepared by cultivating cells o/n at 30°C in liquid SCD-Ura. Then, cells were collected by centrifugation, washed with water and finally resuspended (OD₆₀₀ = 1.0) in 12°C-pre-chilled YP containing maltose as sole carbon source (YPM).

Eighty ml of the yeast mixture was poured into a screw-cap bottle and placed in a 12°C water bath. CO₂ production was recorded for 42 h in a Fermograph II apparatus (ATTO Co., Ltd., Tokyo, Japan). Values are expressed as ml of CO₂ and represent the total volume of gas produced after 24 or 42 h of fermentation or are shown as the time-course graph of total gas. At least three independent experiments were conducted for each yeast strain.

**RESULTS AND DISCUSSION**

**Screening of multicopy suppressors.** The cold sensitive Trp⁺ *S. cerevisiae* strain CEN.PK2-1C was transformed with a high-copy yeast genomic DNA library. In order to prevent excessive basal growth, the screening was carried out at 12°C on complex YPD medium containing 25 μM of phytosphingosine (PHS). Addition of PHS to yeast cells promotes cellular stress responses (19), among them the stimulation of ubiquitin-dependent turnover of amino acid transporters (11, 13, 56). Thus, exposure to this
sphingoid long-chain base restricts further yeast cell growth at low temperature, an
effect that can be counteracted by increased tryptophan availability in the culture
medium or overexpression of the high affinity tryptophan transporter Tat2p (56).

Twenty-eight transformants were initially selected, from which only four were
reconfirmed as showing enhanced growth at 12°C. These were further analyzed by
plasmid isolation and compared on the basis of their restriction map, which revealed
four different patterns (data not shown). A representative of each group (referred to as
pSGM-2/-4/-10/-25) was used to retransform the S. cerevisiae CEN.PK2-1C strain. As
can be seen in Fig. 1A, all the plasmids tested were able to confer improved cold growth
on a synthetic SCD medium lacking PHS, whereas no effect was detected in the
presence of PHS at 30°C. Thus, the screening appeared to select for low temperature-
sensitive suppressors. However, all transformants grew as well as the control strain on
rich YPD medium at 12°C (Fig. 1A). Therefore, it seems that the 12°C-growth
advantage provided by the multicopy suppressors is only effective under conditions of
reduced nutrient availability.

Subsequently, the effect of the aforementioned plasmids was analyzed in a different
genetic background, specifically in the tryptophan prototroph yeast strain BY4741.
TRP1 or tryptophan transporters like TAT2 (52) are most likely the gene(s) responsible
for the observed cold phenotype in the CEN.PK2-1C transformants (Fig. 1A).
Accordingly, growth inhibition at low temperature should not be alleviated in Trp⁺ cells.
Indeed, this was the case for cells containing plasmid pSGM-25 (Fig. 1B), which,
therefore, was not studied further. However, high-copy number of pSGM-2, pSGM-4
and pSGM-10 still conferred some growth improvement in cold-exposed cells of the
wild-type BY4741 strain (Fig. 1B).
**Characterization of the isolated plasmids.** Sequencing of the insert contained in plasmids pSGM-2, pSGM-4 and pSGM-10 revealed the presence of DNA fragments of 9.5-Kb, 8.7-Kb and 8.9-Kb in length from chromosomes X, V and II, respectively. In total, 11 complete and 3 partial ORFs were identified. The former were PCR-amplified from yeast genomic DNA, ligated into the YEplac195 multicopy expression vector, and retransformed into the *S. cerevisiae* CEN.PK2-1C wild-type strain. This led to the identification of *ALY2* (*ART3/YJL084C*), *CAJ1* (*YER048C*) and *UBP13* (*YBL0621*) as responsible for the cold-tolerant phenotype found in pSGM-2, pSGM-4 and pSGM-10 transformants, respectively.

**The endocytic adaptor Aly2p.** Aly2p/Art3p belongs to the ARTs (for arrestin-related trafficking adaptors), a protein family in yeast (32), composed of nine members (44). Like other ART proteins, Aly2p is predicted to contain N-terminal (pfam00339) and C-terminal (pfam02752) arrestin fold domains (structural elements also found in the mammalian α- and β-arrestin family; see ref. 6), and two PY motifs, this being the conserved sequence recognized by the Rsp5p/Npi1p ubiquitin ligase (9). It has been proposed that members of the ART family function as adaptor proteins for Rsp5p, promoting endocytosis of plasma membrane associated proteins (cargoes) in response to environmental cues, or by targeting damaged or superfluous plasma membrane proteins for degradation (32). Recently, Aly2p has been shown to mediate endocytosis of the aspartic acid/glutamic acid trasporter Dip5p by recruitment of Rsp5p (26). Consistent with all of this, lack of Rsp5p (Fig. 2A and 2B) resulted in increased growth at 12°C, whereas Aly2p-overexpression in this strain did not increase cold-growth above the RSP5-deletion mediated rate (Fig. 2A). Furthermore, the absence of a functional Aly2 protein had no major effects on cold-growth (Fig. 2B). Similar results were found for *aly2Δ* mutants of either CEN.PK2-1C (Fig. 2B) or BY4741 (data not shown).
background. Since ARTs have been proposed to confer specificity to endocytosis (44), overproduction of Aly2p might improperly recruit Rsp5p to substrates that are not relevant for low temperature growth, decreasing endocytosis of essential permeases under this condition. In this respect, a recent study by O'Donnell et al. (43) has demonstrated that Aly2p stimulates the trafficking of Gap1p, the general amino acid permease, from endosomes to the trans-Golgi network, thus increasing the level of the transporter within cells and at the plasma membrane.

The J protein Caj1. CAJ1 encodes a member of the cytosolic class II J proteins (also known as Hsp40s), which are defined by the presence of a ~65-AAs J domain (pfam00226), formerly identified in the bacterial heat-shock gene DnaJ (45, 62). All of them are obligate co-chaperones of 70-KDa heat-shock proteins, Hsp70s, stimulating their ATPase activity and thus allowing them to function in multiple cellular processes (30, 49). Specifically, Caj1p was isolated from a yeast calmodulin-binding protein fraction (40). The Ca\(^{2+}\)-regulatory protein calmodulin, controls numerous targets (65), among them the serine/threonine protein phosphatase calcineurin (15), which has, in turn, been found to down-regulate amino acid transporter endocytosis, triggered by several stress conditions (11). Accordingly, expression in a high-copy number of CAJ1 might, somehow, increase calcineurin activity, thus reducing the cold-instigated turnover of transporters and membrane-anchored proteins. To test this hypothesis, CAJ1 transformants of cna1Δ cna2Δ, cnb1Δ and crz1Δ mutant strains (16, 57) were examined for growth at 12°C. In its native form, calcineurin is present as a heterodimer containing a catalytic subunit, encoded by the functionally redundant genes CNA1 and CNA2, complexed with a regulatory subunit, the gene product of CNB1. Active calcineurin dephosphorylates the transcriptional factor Crz1p (36, 57), which regulates the expression of most salt-responsive genes (66). However, high-copy number of CAJ1
increased cold-growth independently of calcineurin-Crz1p pathway functioning (Fig. S1). Furthermore, CAJ1 had no effect on yeast sensitivity to Mn²⁺ cations, a well known stressor triggering this signaling route.

Then, the growth of YEpCAJ1 transformants of the \( \text{rsp}5\Delta \) mutant strain was tested at 12°C. Overproduction of Caj1p has been reported to confer growth resistance to the drug FTY720, an immunosuppressive agent that inhibits amino acid transport in \( S. \text{cerevisiae} \) (63). Although the mechanism of Caj1p-mediated FTY720-toxicity suppression is unknown, it has been hypothesized that this J protein might influence amino acid uptake and/or protein degradation (63). In this respect, tryptophan transporters Tat1p and Tat2p, and ubiquitin proteases Ubp5p and Ubp11p, have also been isolated as multicopy suppressors of FTY720-induced growth inhibition (63). As can be seen in Fig. 2A, the effect of Caj1p at 12°C was completely masked by the absence of a functional Rsp5p ubiquitin-ligase. Hence, our results suggest that Caj1p could play a specific role in modulating the activity of Hsp70s chaperones in the ubiquitin-mediated reprogramming of proteins at low temperature.

**The Ubiquitin C-terminal hydrolase UBP13.** The protease Ubp13p is a member of the family of deubiquitinating enzymes (Dubs), which comprises 17 potential genes in \( S. \text{cerevisiae} \) (7). Dubs are ubiquitin-specific proteases, which release the peptide from ubiquitin-conjugated cargoes, reverting thus the ubiquitination process. As a consequence, Dubs are required for both ubiquitin homeostasis and proteasome-dependent proteolysis (31, 41). In particular, it has been shown that three Dubs, \( \text{DOA4} \), \( \text{UBP6} \) and \( \text{UBP14} \) are involved in pressure-induced degradation of Tat2p since a lack of any of these proteins causes stabilization of the tryptophan transporter and allows yeast cells to grow at high pressure (38). In contrast, we found that overexpression of \( \text{UBP13} \) was beneficial to cells at low temperature, whereas its absence had no effect on growth.
at 12°C (data not shown). Moreover, these effects were again dependent on the existence of a functional Rsp5 protein (Fig. 2A). In this context, it is possible that Ubp13p might have either specific or overlapping functions at low temperature, as previously suggested for other deubiquitinating enzymes (7).

Cold regulation of membrane transporters and functional role of Rsp5p. The above results would suggest that cold is perceived as a stress condition for membrane transporter degradation and that Rsp5p might be involved in this process. To test this, first the levels of general amino acid permease Gap1p were analyzed in cells transformed with plasmid YCpJ25, containing the GAL1-promoter dependent GFP-tagged GAP1 gene (18). As can be seen, the amount of Gap1p decreased when cells of the wild-type strain CEN.PK2-1C were transferred from 30 to 12°C at 4 h (Fig. 3A).

Then, the level of Tat1p and Tat2p was examined in cells of the wild-type and rsp5Δ mutant cells transformed with plasmid p3HA-TAT1c or p3HA-TAT2c, each encoding an N-terminally hemagglutinin (HA)-tagged, fully functional Tat1 or Tat2 protein (1, 2). As expected, the level of both tryptophan permeases was higher in cells of the rsp5Δ mutant grown at 30°C than in the corresponding parental strain (Fig. 3B and 3C). Exposure to low temperature caused a reduction in Tat1p in both wild-type and mutant cells, suggesting that another ubiquitin ligase may control the regulation of Tat1p in response to low temperature, as reported for yeast cells subjected to high hydrostatic pressure (2). Nevertheless, cold-instigated changes in the level of Tat1p (Fig. 3B), such as those shown in Gap1p (Fig. 3A), were scarce after 4 h of exposure to low temperature. To examine whether a longer incubation period at 12°C can cause stronger effects, 3HA-Tat2p cells were analyzed after 24 h of cold-treatment (OD600 ~ 0.4-0.5). As shown, long-term exposure to low temperature strongly reduced the level of Tat2p in wild-type cells (Fig. 3C). However, this change was less pronounced in rsp5Δ mutant
cells, which still showed an intense Tat2p-band after 24 h at 12°C (Fig. 3C). Hence, cold exposure of yeast cells triggers the downregulation of various membrane permeases and Rsp5p plays a role in this mechanism.

**Overexpression of ALY2 increases Tat2p abundance, which is essential in the cold-growth of yeast cells.** We tried to further clarify the involvement of tryptophan permeases in the cold-sensitivity suppression mechanism. The study was restricted to the effects mediated by ALY2 and UBP13 because LEU2-based plasmids containing CAJ1 could not be obtained. First, we analyzed whether the improved cold-growth was dependent on the presence of a functional Tat1p or Tat2p permease. CEN.PK2-1C mutant strains lacking TAT1 or TAT2 were constructed and transformed with plasmids for ALY2 or UBP13. As shown, deletion of either of these tryptophan permeases prevented cell proliferation at 12°C under conditions of limited tryptophan availability, SCD medium (Fig. 4A). A high-expression of ALY2 or UBP13 conferred cold-growth, but this effect was much more pronounced in a strain lacking TAT1 than in the tat2Δ mutant.

Then, we examined whether the level of Tat2p was affected by overexpression of ALY2 or UBP13. As a control, the levels of Gap1p were also checked. LEU2-based plasmids carrying the multicopy suppressors were used to transform p3HA-TAT2c- and YCpJ25-derivatives of the CEN.PK2-1C strain. As can be seen in Fig. 4B, neither ALY2 nor UBP13 appeared to affect the level of Tat2p in cells grown at 30°C. Overexpression of UBP13 had limited effects, if any, in the abundance of Tat2p at 12°C. However, a high expression of ALY2 seemed to inhibit the cold-stimulated Tat2p degradation since Tat2p levels remained stable after 24 h at 12°C (Fig. 4B). ALY2 overexpression also increased Gap1p levels, although its effects were already evident at 30°C (Fig. 4B), whereas UBP13 caused no changes, or even appeared to reduce the abundance of the
permease in cells grown at 12ºC (Fig. 4B). Overall, our results suggests that down-regulation of Tat2p is the main cause for yeast growth inhibition at low temperature. Overexpression of \textit{ALY2} counteracts the cold-instigated down-regulation of Tat2p and increases the relative content of Gap1p, although other plasma membrane transporters must also be considered to fully explain the ability of \textit{ALY2}- and in particular \textit{UBP13}-transformants to grow under low-temperature conditions.

Enhanced growth of industrial strains at low temperature. We were interested in investigating whether increasing gene dosage of cold-sensitivity suppressors might be a useful approach to improve growth at low temperature of industrial strains. To address this, first Ura’ and Ura’ Trp’ auxotrophic derivatives of the QA23 wine strain were selected. Single and double mutants were transformed with \textit{URA3}-based plasmids YEpALY2, YEpCAJ1 or YEpUBP13 and tested for growth at low temperature. As expected, enhanced growth at 12ºC was only observed for Trp’ transformants, but not for prototroph strains (data not shown). Thus, the ability of these three genes to increase yeast growth at 12ºC appeared to be closely related to their capacity to interfere in the cold-instigated regulation of amino acid transporters. At this point, the question arose as to whether this property may be extended to other nutrient permeases and, in that case, under which conditions the overexpression of \textit{ALY2}, \textit{CAJ1} or \textit{UBP13} could confer cold-growth advantages in prototroph industrial strains.

An important feature of the \textit{S. cerevisiae} genome is the presence of many glucose transporters. Indeed, at least 17 genes need to be deleted, \textit{HXT1-17}, to ensure that \textit{Saccharomyces} is unable to take up and to grow on glucose as sole carbon source (64). Conversely, maltose is internalized in most \textit{S. cerevisiae} strains by single genes, \textit{MAL31/MAL61}. Furthermore, yeast maltose transport is regulated by catabolite inactivation through Rsp5p and Doa4p-mediated proteolysis (34). As shown in Fig. 5A,
overexpression of \textit{ALY2} and \textit{UBP13} conferred enhanced growth at 12\textdegree C to prototroph cells of the wine strain QA23 cultivated on SC with maltose as sole carbon source (SCM), whereas no differences could be found at 30\textdegree C. Similar results were observed in rich medium YP supplemented with maltose (YPM, Fig. 5A). No effects were observed in \textit{YEPCAJ1} transformants, suggesting that this J-protein alters targets other than maltose permease.

Thus, the gassing power of the recombinant strains was inspected. As can be seen in Fig. 5B (upper graph), the rate of CO\textsubscript{2} production by \textit{UBP13}, and specially \textit{ALY2} transformants, was clearly higher than that recorded for \textit{YEPCAJ1} or control cells in liquid YPM at 12\textdegree C. Thus, CO\textsubscript{2} production attained by overexpression of \textit{ALY2} after 42 h of fermentation at low temperature was about 30\% higher than that observed with the control strain under these conditions (Fig. 5B, lower graph).

\textbf{CONCLUDING REMARKS}

Our work has identified low-temperature sensitivity suppressors in \textit{S. cerevisiae} for the first time. Since nutrient permeases are essential for cold-growth, it was expected that this approach would identify, among others, genes involved in regulating the membrane protein repertoire and activity. Indeed, three poorly characterized genes (\textit{ALY2}, \textit{CAJ1} and \textit{UBP13}) were found to provide enhanced growth at 12\textdegree C. Of these, only \textit{ALY2} has been studied in greater depth recently (26, 43). According to these reports, the alpha-arrestin Aly2p mediates the endocytosis of Dip5 (26), and regulates the trafficking of Gap1p in response to nutrient signaling (43). Indeed, based on our analysis, not only Aly2p, but also Caj1p and Ubp13p, comprise or are involved in the
activity of the ubiquitin machinery. This machinery controls membrane protein trafficking in yeast, with the ubiquitin ligase Rsp5p as a major player in this regulatory system. Our analysis demonstrated that tryptophan permeases, in particular Tat2p, but also other transporters like Gap1p, are down-regulated at low temperature and that their steady state level is controlled by Rsp5p. Furthermore, we also showed that Aly2p inhibits the cold-instigated down-regulation of Tat2p and increases the abundance of Gap1p. Nevertheless, further studies are required to understand and clarify the exact suppressor mechanism for Aly2p, Caj1p and Ubp13p. Since many other genes have been identified as being involved in the plasma membrane transporter regulation at different steps, it is to be expected that more cold-suppressors may be identified. This would help to elucidate the exact machinery operating at low temperatures and identify new candidates providing increased growth under this restrictive condition. In light of our results, this has indeed, potential biotechnological applications to improve growth and activity of industrial strains under nutrient-limiting conditions.

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REFERENCES


Figure Legends

FIG. 1. Cold-sensitivity suppressors provide enhanced growth at 12°C under conditions of reduced nutrient availability. (A) Tryptophan auxotrophic (trp1) cells of the *S. cerevisiae* CEN.PK2-1C wild-type strain were transformed with plasmids pSGM-2, pSGM-4, pSGM-10 and pSGM-25, all of which were isolated from the screening of a high-copy yeast genomic DNA library for cold-sensitivity suppressors, and transformants were assayed for growth at 30°C or 12°C. Cells were pre-grown in SCD liquid medium at 30°C until the early-exponential phase and adjusted to OD$_{600} = 0.8$. Serial dilutions (1-10$^{-3}$) of the adjusted cultures were spotted (3 µl) onto standard SCD-Ura, YPD or YPD containing 25 µM of phytosphingosine (PHS) solid medium. Cells transformed with plasmid YEplac195 (*URA3*, empty plasmid) were used as control. (B) Transformants of the Trp$^+$ BY4741 wild-type strain were cultivated at 30°C or 12°C on SCD-Ura plates as indicated above. In all cases, a representative experiment is shown.

FIG. 2. The effect of cold-sensitivity suppressors Aly2p, Caj1p and Ubp13p depends on the existence of a functional ubiquitin ligase Rsp5p. (A) Cells of the *rsp5*Δ mutant strain and its corresponding parental (Σ1278b) were transformed with YEplac195 (*URA3*)-based plasmids containing the whole ORFs for *ALY2*, *CAJ1* and *UBP13* including its own promoter and terminator, and tested for growth on SCD-Ura solid medium at 30 or 12°C. (B) Growth of *rsp5*Δ and *aly2*Δ mutant cells and their corresponding wild-type strains, Σ1278b and CEN.PK2-1C, respectively, was examined on rich YPD and minimal SCD medium. In all cases, cells were pre-grown and spotted onto solid medium plates as described in Fig.1. In all cases, a representative experiment is shown.
FIG. 3. The ubiquitin ligase Rsp5 influences the steady state level of different plasma membrane permeases, which are downregulated in response to low temperature. (A) CEN.PK2-1C cells transformed with plasmid YCpJ25 (18), were pre-grown overnight in SCD, and then refreshed (OD$_{600}$ ~ 0.25) in SCG (90 min), containing galactose as sole carbon source. Then, 2% glucose was added and the culture was transfer to a 12°C-chilled water bath for the times indicated. Preparation of protein extracts, SDS-PAGE separation and visualization of Gap1-GFP and Hxk2p (loading control) were performed as described in the Materials and Methods section. A protein extract from untransformed cells was used as negative control (C-). (B) Cells of the $rsp5\Delta$ mutant strain and its corresponding parental ($\Sigma1278b$) carrying a 3HA-TAT1c plasmid (2) were grown in SCD-Ura, refreshed in the same medium and incubated at 30°C until the culture reached an OD$_{600}$ ~ 0.5 or at 12°C for the periods indicated. Cells were processed and protein extracts analyzed as described above, except that Tat1p was detected using an anti-HA rabbit polyclonal antibody. (C) The same strains as in (B) were transformed with plasmid 3HA-TAT2c (2) and protein extracts were analyzed as above, except that the cold treatment was extended to a 24-h period. In all cases, a representative experiment is shown.

FIG. 4. A high-copy number of $ALY2$ reduces the cold-induced down-regulation of the tryptophan transporter Tat2p, which is important for cold-growth. (A) YEplac181 (Control, $LEU2$), YEp181ALY2 ($ALY2$) and YEp181UBP13 ($UBP13$) transformants of $tat1\Delta$ and $tat2\Delta$ mutants of the $S. cerevisiae$ CEN.PK2-1C background were examined for growth at 30 or 12°C. YEplac181 transformants (Control) of the wild-type (wt) strain were also spotted as reference. Cells were pre-grown in liquid
SCD-Leu and then the cultures were diluted and spotted onto solid medium as described in the Fig. 1. (B) Cell lysates were subjected to SDS-PAGE and immunoblotted with anti-HA or anti-HxK2 antibody. Analysis was performed of CEN.PK2-1C-derivative strains co-transformed with plasmids 3HA-TAT2c (URA3) and YEplac181 (Control, LEU2), YEp181ALY2 (ALY2) or YEp181UBP13 (UBP13). Cells were pre-grown at 30°C and protein extracts were treated as described in Fig. 3C. (C) The levels of the general amino acid permease Gap1p were analyzed in cells co-transformed with plasmid YCpJ25, which contains the GAL1-promoter dependent GFP-tagged GAP1 gene (18), and YEplac181 (Control, LEU2), YEp181ALY2 (ALY2) or YEp181UBP13 (UBP13). Cells were grown as described in Fig. 3A, except that the cold treatment was extended to a 24-h period. Preparation of protein extracts, SDS-PAGE separation and visualization of Gap1-GFP and Hxk2p (loading control) were performed as described in the Materials and Methods section. In all cases, a representative experiment is shown.

FIG. 5. Engineering of ALY2 or UBP13 improves the cold-growth and fermentative activity of the prototroph industrial QA23 strain on maltose. (A) Ura- derivatives of the wine strain QA23 ho were transformed with YEplac195-based plasmids (URA3) containing ALY2, CAJ1 or UBP13 and assayed for growth at 30 or 12°C on SC medium containing glucose (SCD-Ura) or maltose (SCM-Ura) as sole carbon source, or on rich YP-maltose (YPM). Transformants carrying the empty plasmid, were also tested (Control). In all cases, cells were pre-grown and treated as described in the Fig.1. In all cases, a representative experiment is shown. (B) The fermentative performance of the mentioned strains was tested on liquid YPM at 12°C by measuring the CO2 production in a fermograph apparatus. Cell biomass was prepared and CO2 was measured as described in the Materials and Methods section. Values are expressed as ml of CO2 and
represent the temporal change graph of total gas (upper graph) or the total volume of gas produced after 24 or 42 h of fermentation (lower graph). At least three independent experiments were conducted for each yeast strain. The error (lower graph) was calculated using the formula: \((1.96 \times SD) / \sqrt{n}\), where \(n\) is the number of measurements.
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FIG. 1
FIG. 2
FIG. 3
FIG. 4