Post-translational Modification of Plant Plasma Membrane H^+-ATPase as a Requirement for Functional Complementation of a Yeast Transport Mutant*

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Many heterologous membrane proteins expressed in the yeast Saccharomyces cerevisiae fail to reach their normal cellular location and instead accumulate in stacked internal membranes. Arabidopsis thaliana plasma membrane H^+-ATPase isoform 2 (AHA2) is expressed predominantly in yeast internal membranes and fails to complement a yeast strain devoid of its endogenous H^+-ATPase Pma1. We observed that phosphorylation of AHA2 in the heterologous host and subsequent binding of 14-3-3 protein at the penultimate residue (Thr^947), which creates a binding site for endogenous 14-3-3 protein. Only a pool of ATPase in the plasma membrane is phosphorylated. Double mutants carrying in addition a T947A substitution lost their ability to complement Pma1. Thus, mutants of AHA2, complementing pma1, showed increased phosphorylation at the penultimate residue (Thr^947), which creates a binding site for endogenous 14-3-3 protein. Double mutants carrying in addition a T947A substitution lost their ability to complement pma1. However, mutants affected in both autoinhibitory regions of the C-terminal regulatory domain complemented pma1 irrespective of their ability to become phosphorylated at Thr^947. This demonstrates that it is the activity status of the mutant enzyme and neither redirection of trafficking nor 14-3-3 binding per se that determines the ability of H^+-pumps to rescue pma1.

The yeast Saccharomyces cerevisiae has been used for many years as a heterologous system to assign functions to membrane proteins. Because yeast genes are easily disrupted, endogenous transport activities can be suppressed, which in several cases renders the yeast strain dependent on the transgene for growth. However, in a number of cases the recombinant membrane protein was found to be retained in the endoplasmic reticulum (ER), Golgi apparatus, or secretory vesicles and did not reach its target membrane in sufficient amounts (1–7). Thus, it has been speculated that the deficiency in complementation of yeast transport mutants could be caused by targeting problems in this heterologous host (7).

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The abbreviations used are: ER, endoplasmic reticulum; AHA2, Arabidopsis thaliana plasma membrane H^+-ATPase isoform 2; GFP, green fluorescent protein; EGFP, enhanced green fluorescent protein.

Plasma membrane H^+-ATPases, which catalyze the ejection of protons from the cytosol into the apoplast at the expense of ATP, belong to the superfamily of P-type ATPases, cation pumps that comprise a single catalytic subunit with a phosphorylated intermediate as part of their reaction cycle. Plant plasma membrane H^+-ATPase is an example of a protein that can be expressed in yeast to high levels in a functional form but often fails to replace its yeast counterpart, Pma1 (7, 8). However, a large number of mutations in plant plasma membrane H^+-ATPases that result in the ability of those pumps to rescue pma1 mutants have been identified (9–12). Most of these mutants have been localized to the C-terminal cytosolic domain, which comprises about 100 amino acid residues following the last transmembrane span (M10). Recently, an alanine-scanning mutagenesis through the C-terminal 87 amino acid residues of the plant plasma membrane H^+-ATPase AHA2 (Arabidopsis thaliana H^+-ATPase isoform 2) revealed a total of 32 mutant enzymes that when expressed in yeast complemented a defect in the endogenous PMA1 (12). The mutants cluster in two regions, I and II, stretching from Lys^963 to Leu^985 and Ser^904 to Leu^919, respectively.

The C-terminal regulatory domain of the H^+-pump (the R-domain) is an autoinhibitory domain that interacts directly with regulatory protein 14-3-3. As a result of this interaction, the H^+-ATPase acquires an activated state characterized by an increase in V_max, an increased affinity for ATP, and a change in pH dependence with higher activity toward physiological pH (13). The site of interaction of 14-3-3 protein with the plant H^+-ATPase has been mapped to the extreme C-terminal end of the pump (14–16). Binding has been shown to involve the three C-terminal residues (Y946TV in AHA2) and requires phosphorylation of the penultimate residue (Thr^947 in AHA2).

In the present study we show that regions I and II are true autoinhibitory sequences and that both regions have to be neutralized concomitantly for complementation of pma1 to be effective. However, phosphorylation at Thr^947 of AHA2 and subsequent binding of 14-3-3 protein, events that can be promoted by single point mutations, also lead to full activation of pump activity by a mechanism, which most likely involves disruption of an interaction between the two autoinhibitory regions and the rest of the H^+-ATPase. This post-translational modification apparently is a prerequisite for the ability of this plant transporter to replace its yeast counterpart. We suggest that similar post-translational modifications are required for plasma membrane H^+-ATPases to function properly in planta.

**EXPERIMENTAL PROCEDURES**

**Construction of Mutants**—The multicopy vector YEp-351 (17) containing the full-length cDNA of AHA2 under control of the PMA1 promoter (7) was employed. Site-directed mutagenesis to generate alanine mutants was performed previously (12). Double point mutants,
Green Fluorescent Protein Constructs—The AHA2 H⁺-ATPase (plasmid pMP568) was tagged by introducing an enhanced green fluorescent protein (EGFP) to its N-terminal end (resulting in plasmid pMP1000). To generate EGFP, the P44L mutation (18) was introduced into sGFP (S65T) using a PCR-based approach. A plasmid harboring the cDNA encoding sGFP (S65T) was obtained as a generous gift from Dr. Sheen (19). A SacII restriction site was introduced upstream of both the EGFP stop codon and the AHA2 start codon using a PCR-based approach, without disturbing the reading frames. The EGFP sequence was inserted between the XhoI restriction site and the newly created SacII restriction site of pMP568. As a result of the construct, an SAA tripeptide linker was inserted between the two components of the EGFP-AHA2 fusion protein, and also a CGG CCA GAA CA sequence was inserted between the start codon of EGFP and the XhoI restriction site of the pMP658 plasmid.

Expression in the Yeast S. cerevisiae—RS-72 (MATa ade1-100 his4–519 leu2–3,112) was transformed and cultured essentially as described previously (20). In RS-72, the natural constitutive promoter of the endogenous yeast plasma membrane H⁺-ATPase PMA1 has been replaced by the galactose-dependent promoter of GAL1. Because PMA1 is essential for yeast growth, this strain only grows on galactose medium. Alternatively, AHA2 and its mutants were transformed into a yeast strain deleted in the 14-3-3 protein homologues BMH1 and BMH2 (RRY1216, a kind gift from Dr. G. Fink). The cells were grown and harvested, and plasma membranes were isolated on a sucrose step gradient at the 43/53% (w/w) interface as described (20). Fractionation of yeast microsomal membranes on a linear sucrose density gradient (33–55% w/w) was as described (7).

ATPase Assay—ATPase activity was determined as described (20). Gel Electrophoresis and Western Blotting—Plasma membrane proteins were separated by SDS-PAGE using 10% polyacrylamide gels and the buffer system of Fling and Gregerson (21). After electrotransfer of the proteins to a Protran nitrocellulose transfer membrane (Schleicher & Schuell), proteins were probed with an antibody raised against the H⁺-ATPase Pma1. It has been suggested that this is an antibody against a barley 14-3-3 protein. Alternatively, membranes were probed with 14-3-3 protein fused to cyan fluorescent protein for overlay assay as described earlier (14).

Growth Test—Yeast was grown on solid, synthetic media containing 2% galactose as the only carbon source and supplemented with adenine and histidine. 10⁵ cells in 10 μl were spotted onto fresh plates containing either galactose at pH 5.5 or glucose at pH 5.5 or 4.5. Growth was recorded after incubation at 30 °C for 2 to 3 days.

Immunolocalization and Confocal Microscopy—Yeast cells were fixed in 5% freshly made paraformaldehyde in culture medium for 15 min, gently spun down (5 min at 3000 rpm), and resuspended in 5% paraformaldehyde in buffer A (50 mM K₂HPO₄, KH₂PO₄, 0.05 mM MgCl₂, and 1.2 mM sorbitol, pH 6.5) for 30 min. After washing in buffer B (buffer A with mercaptoethanol, pH 7.5), the cell walls were removed by treatment in 1% (v/v) proteinase K (buffer C) at 37 °C for 30 min. After washing in buffer B the cells were mounted on polylysine-coated microscope slides (Teflon-printed multowell slides) and air-dried. Cell membranes were permeated and denaturated by immersing the slides in absolute methanol at –20 °C for 6 min and in aceton at –20 °C acetone for 30 s. The slides were incubated in the primary monoclonal antibody (1:150) in PBS-BSA (phosphate-buffered saline-bovine serum albumin) for 30 min, washed with PBS-BSA, incubated with secondary IgG anti-mouse (diluted with Alexa 488 (1:50) in PBS-BSA for 30 min, and washed with PBS-BSA. The cells were mounted with anti-bleach medium (1,4-diazabicyclo[2.2.2]octane) and stored in the dark under refrigeration.

Observation and documentation with the confocal laser scanning microscopy was with fluorescein isothiocyanate filter setting (laser at 488 nm, beam splitter at 510 nm, and emission filter at 520–580 nm BP). For each mutant a complete stack of optical sections was recorded to find the median section where peripheral and internal localization of the antibody could be discriminated.

RESULTS

Distribution of Arabidopsis AHA2 in the Heterologous Host, S. cerevisiae—Plant plasma membrane H⁺-ATPases often fail to complement mutations in the endogenous yeast plasma membrane H⁺-ATPase Pma1. It has been suggested that this is due to a targeting defect because, following expression in yeast, plant plasma membrane H⁺-ATPases such as AHA1 (7) and AHA2 (20) accumulate in internal membranes. However, a small fraction of AHA2 can be immunolocalized close to or within the plasma membrane (20) suggesting that at least a fraction of the ATPase is able to reach this location.

Using an improved method for immunolocalization, we localized AHA2 protein to the plasma membrane in addition to internal membrane structures (Fig. 1A). In the plasma membrane, part of the fluorescence was organized in what appeared to be a punctate pattern. To confirm these findings, we constructed an in-frame fusion between AHA2 and green fluorescent protein and expressed the fusion protein in yeast. Fluorescence was intense in internal membranes surrounding the nucleus, an indication of ER localization (Fig. 2). Furthermore, peripheral fluorescence, indicating plasma membrane localization, was clearly evident and partially patch-like (Fig. 2). This indicates that use of the immunofluorescence technique revealed the correct localization of the recombinant protein expressed in yeast.

Class C Mutants Show a Similar Intracellular Distribution as AHA2—Class C mutants of AHA2 are defined such that they readily complement pma1 (12). We analyzed in more detail a corresponding mutant protein that carries a substitution to alanine at position Arg¹³. Despite its ability to substitute for the endogenous yeast proton pump (Fig. 3), the aha2R913A mutant protein Pma1 showed a very similar intracellular and peripheral localization when compared with AHA2 (Fig. 1B). The same localization pattern was also assessed for mutant aha2T947A (Fig. 1C) and a double mutant aha2R913A,T947A (Fig. 1D), which were both impaired in binding the 14-3-3 protein and unable to substitute for Pma1 (Fig. 3).
AHA2, the pH dependence of aha2R913A and other class C mutant enzymes is shifted with higher activity toward neutral pH (12) (Fig. 4A). This kinetic profile is typical for the plasma membrane H⁺-ATPase activated via displacement of its C-terminal autoinhibitory domain (13) and might indicate a correlation between the activatory status of the plant enzyme and its ability to substitute for Pma1.

To test this hypothesis, we analyzed the activation state in a number of AHA2 deletion mutants with different ability to substitute for Pma1. Deletion of 3, 10, 30, or 40 amino acid residues had no positive effect on the ability to replace Pma1, whereas deletion of 92 residues fully restored the growth of the yeast strain (Fig. 5A). Sucrose density gradient centrifugation of microsomal membranes followed by immunological quantification of AHA2 protein in the various fractions revealed that all deletion mutants were expressed to about the same level in the yeast plasma membrane (data not shown).

Next, we used the shift in pH optimum as an indicative of AHA2 activation. Deletion of just 3 or 10 C-terminal amino acid residues did not lead to a changed pH profile compared with AHA2, whereas deletion of 30 or 40 C-terminal amino acids gave rise to a significant shift in the pH dependence of ATP hydrolysis (Fig. 5B) similar to that seen in the complementing mutant, aha2R913A (Fig. 4A). Deletion of 92 C-terminal amino acid residues resulted in an additional shift of the activity profile toward alkaline pH.

The data given above demonstrate that it is possible to generate regulatory mutants of AHA2 that appear to be activated but, nevertheless, are not able to replace Pma1.

Class C Mutants of AHA2 Have Increased Binding of 14-3-3 Proteins—

14-3-3 proteins have been implicated in the activation of plant plasma membrane H⁺-ATPases. To investigate the possibility that binding of endogenous yeast 14-3-3 protein might be involved in the activation of mutants of AHA2 expressed in yeast, plasma membrane-enriched fractions of yeast expressing representatives of each class of mutants were prepared. 14-3-3 proteins are soluble and therefore not normally associated with the yeast plasma membrane. Accordingly, plasma membranes from cells expressing either no ATPase or AHA2 contained little immunodetectable 14-3-3 protein (Fig. 6B). In contrast, membranes expressing class C mutants contained significant amounts of immunodetectable 14-3-3 protein (Fig. 6B). Class B mutants, showing a somewhat intermediate complementation efficiency, had increased binding of 14-3-3 protein compared with AHA2 but not to the same degree as class C mutants (Fig. 6B).

Class C Mutants of AHA2 are Phosphorylated at Thr⁹⁴⁷—It has recently been shown that binding of 14-3-3 protein to AHA2.
FIG. 5. Effect of C-terminal deletions on AHA2 expressed in yeast. A, growth phenotype of a pma1 mutant of yeast transformed with various C-terminal deletion mutants of AHA2 plasma membrane H^+-ATPase. Each strain was grown under three different conditions: galactose medium at pH 5.5 and glucose medium at pH 5.5 and 4.5. G, galactose medium; D, glucose medium. B, pH dependence of ATP hydrolysis by AHA2 plasma membrane H^+-ATPase and C-terminal deletion mutants expressed in yeast. Plasma membrane-enriched fractions were employed for the activity measurements. The assay medium contained 3 mM ATP. ○, AHA2; ●, aha2Δ3; □, aha2Δ10; ◇, aha2Δ30; ▲, aha2Δ40; and ◆, aha2Δ92.

plasma membrane H^+-ATPase is dependent upon phosphorylation of Thr^947 in AHA2, the penultimate residue (14–15). To test the phosphorylation status of the recombinant H^+-pumps, we used an antibody directed against phosphothreonine (Fig. 6C). AHA2 and class A mutants were only marginally labeled. However, the antibody gave rise to a strong signal with class C mutants, whereas class B mutants were less intensely labeled.

Provided that Thr^947 is phosphorylated, plasma membrane H^+-ATPase will bind 14-3-3 protein directly in an in vitro overlay assay (14). To test whether direct binding of 14-3-3 protein to AHA2 is possible, the nitrocellulose membranes were incubated with 14-3-3 protein fused at the gene level to cyan fluorescent protein (14). Compared with AHA2, class B and C mutants should have increased binding of 14-3-3-cyan fluorescent protein in vitro (Fig. 6D). Binding was further improved by the addition of 5 μM fusicoccin (Fig. 6E), a fungal phytotoxin that is known to stabilize the complex between 14-3-3 protein and H^+-ATPase.

The aha2R913A,T947A mutant was not recognized by a phosphothreonine antibody (Fig. 7B) suggesting that phosphorylation occurred at position Thr^947. Mass spectrometry analysis (14) confirmed that Thr^947 was phosphorylated in gel purified aha2R913A (data not shown). No other phosphorylated residues could be detected within the C-terminal domain of the mutant enzyme.

Together, this suggests: (i) a direct interaction of the yeast 14-3-3 proteins with aha2R913A and respective mutants; (ii) that this interaction was the result of a phosphorylation of aha2R913A at Thr^947; (iii) that the fraction of H^+-ATPase molecules phosphorylated was significantly higher in aha2R913A and other class C mutants compared with class A mutants and the AHA2 protein; and (iv) that the increased ability to bind 14-3-3 protein of different point mutations correlates directly with their ability to substitute for Pma1.

Phosphorylation of aha2R913A Occurs Following Exit from Internal Membranes of Yeast—The phosphorylation status of class C mutants might be dependent on their cellular location. To test this possibility, microsomes from yeast expressing either aha2R913A or aha2R913A,T947A were fractionated on sucrose step gradients to partially separate endomembranes from plasma membrane-containing fractions. Only the plasma membrane fraction containing aha2R913A was able to bind 14-3-3 protein and could be recognized by the anti-phosphothreonine antibody (Fig. 7B).

Plasma membrane-enriched microsomes were subsequently run on a linear sucrose gradient between 33 and 55% sucrose to more closely characterize the composition of the vesicle fraction (Fig. 8). In these experiments, yeast cells were allowed to divide only three times on glucose medium before harvest, to ensure that traces of Pma1 were still present in the plasma membrane. From overlay assays of the fractions it appeared that the form of aha2R913A, which is able to bind 14-3-3 protein, was specifically localized in a dense fraction of the gradient likely to represent the plasma membrane since it co-migrated with Pma1. However, aha2R913A protein as such was also localized in lighter membranes, probably representing endomembrane vesicles since it co-migrated with Kar2 (a soluble marker for

FIG. 6. Activated mutants of AHA2 plasma membrane H^+-ATPase expressed in yeast are strongly phosphorylated and bind 14-3-3 protein more efficiently than did AHA2. 15 μg of plasma membrane-enriched microsomes of yeast strains expressing various mutants of AHA2 was run on SDS-PAGE. Proteins were transferred to nitrocellulose for immunostaining or overlay assays. control, yeast strain not expressing any H^+-ATPase.
Functional Complementation of Yeast pma1

Post-translational Modifications of AHA2 Govern Whether This Enzyme Can Replace Its Counterpart in Yeast—The results obtained here suggest that post-translational modifications of the plant H\(^+\)-ATPase in yeast is a crucial component in determining the ability of this pump to substitute for Pma1 in the heterologous host yeast. Following expression of AHA2 and mutant forms of the enzyme, we observed a direct correlation between (i) the degree of phosphorylation of recombinant enzymes, (ii) the amount of 14-3-3 protein interacting with the enzymes, and (iii) the ability of the respective enzyme to support growth of yeast.

The immunolocalization studies on the mutants indicate that the mutations did not interfere with the targeting of the protein during post-translational transport and that, despite accumulation in internal membranes, a fraction of AHA2 was delivered to the yeast plasma membrane. A GFP fusion protein also labeled both an intracellular endomembrane compartment as well as the plasma membrane. In the latter case, patch-like structures were apparent, furthermore, just below the membrane surface. At present it is unclear whether this punctate pattern indicates a local accumulation of the pump in the plasma membrane or reflects the accumulation of vesicles or membrane sheets just below the plasma membrane.

Deletion of only three amino acid residues from the C terminus of AHA2 results in the loss of the phosphorylation site and therefore yields an enzyme that is no longer able to bind 14-3-3 protein (14). Therefore, none of the tested deletion mutants is able to bind 14-3-3 protein. Successive deletion of the C terminus demonstrated that deletion of 30 and 40 amino acids leads to activated enzymes as judged by the shift in optimum pH (Fig. 5A). The deletion of 30 amino acid residues already had resulted in an enzyme with pH dependence similar to that of class C mutants. Further deletion, however, was necessary to produce an enzyme able to substitute for Pma1 to the same extent as class C mutants.

Phosphorylation of the plant H\(^+\)-pump apparently occurs in the plasma membrane of the yeast cells. Phosphorylation and/or the subsequent binding of 14-3-3 protein leads to activation in vivo. Only a fraction of the total amount of AHA2 expressed in yeast is localized in the plasma membrane. Therefore, in a mixed population, the increased activity of phosphorylated pumps able to bind 14-3-3 protein versus nonphosphorylated pumps impaired in binding 14-3-3 protein may be

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**Fig. 7.** AHA2 plasma membrane H\(^+\)-ATPase expressed in yeast is phosphorylated at position Thr\(^{947}\) in the plasma membrane and binds 14-3-3 protein. 15 \(\mu\)g each of plasma membrane-enriched microsomes (43/53) and endomembranes (29/33) of yeast strains expressing aha2R913A and aha2R913A,T947A were run on SDS-PAGE. Proteins were transferred to nitrocellulose membrane for immunostaining or overlay assays.

**Fig. 8.** AHA2 capable of binding 14-3-3 is present in plasma membrane preparations. Two ml of a suspension of plasma membrane-enriched microsomes of a yeast strain expressing aha2R913A was layered onto a linear sucrose gradient from 33-55% sucrose. Frac-
difficult to record. Thus, the amounts of nonphosphorylated pumps in the ER were consistently higher than those of phosphorylated pumps in the plasma membrane.

Our results would suggest that it is not binding of 14-3-3 protein per se that determines the ability of H^+ -ATPase to substitute for Pma1 but rather the changed kinetic profile of the enzyme, which can be brought about by either binding of 14-3-3 protein or neutralization of the autoinhibitory C-terminal domain. This hypothesis was confirmed by producing a mutant enzyme with a destroyed phosphorylation site and with both autoinhibitory regions modified. Although this mutant could not bind 14-3-3 protein and carried no deletions in its C-terminal end, it had an alkaline shift in its pH dependence and readily replaced Pma1.

On the basis of the above results, we suggest that binding of 14-3-3 protein to class C mutants in the plasma membrane results in the additional activation needed to substitute for Pma1 in vivo. Thus it may be possible that the small population of mutant enzymes that is targeted to the plasma membrane, phosphorylated, and bound to 14-3-3 protein shows kinetic properties and pH dependence that are similar to the highly activated deletion mutant aha2Δ92 (20). This would resemble the behavior of tobacco PMA2 expressed in yeast (16).

In planta, it seems likely that phosphorylation and 14-3-3 binding is required for plasma membrane H^+ -ATPases to function to the full extent. In line with this hypothesis it has been demonstrated that 2-fold overexpression of PM4A plasma membrane H^+ -ATPase in tobacco does not lead to detectable phenotypes (22), whereas expression of a C-terminally modified pump leads to increased acid tolerance of transgenic A. thaliana (23).

**Is Phosphorylation and Binding of 14-3-3 Protein to Mutants of AHA2 a Primary or Secondary Effect?**—When mutants from class C were further mutated to destroy the phosphorylation site and their ability to bind 14-3-3 protein, the resulting mutants still revealed a substantial degree of activation, suggesting that the point mutations within region I or II per se resulted in a conformational change independent of 14-3-3 binding. This confirms the suggestion (12) that regions I and II are true autoinhibitory regions. It seems likely that this conformational change is the primary effect, which allows for phosphorylation of Thr^347 via a plasma membrane-bound yeast kinase and subsequent binding of 14-3-3 protein.

Based on site-directed mutagenesis of Q943P, Q944S within tobacco H^+ -ATPase PMA2, it has been suggested (16) that AHA2, which lacks the QQ motif but possesses the PS sequence, may not serve as the substrate for the yeast protein kinase responsible for phosphorylation of Thr^347. As a consequence, AHA2, in contrast to PMA2, would fail to substitute for the yeast endogenous H^+ pump. This reasoning, however, is not in line with our finding that mutations upstream of the phosphorylation motif can result in an enzyme with increased phosphorylation at Thr^347. Our data suggest rather that when AHA2 is expressed in yeast, Thr^347 is not exposed to the protein kinase unless at least one of the two autoinhibitory regions is mutated.

One possible interpretation is that the major pool of AHA2 is not properly folded when expressed in the heterologous host yeast and that mutations within the C-terminal domain by some means allow for a better folding, which is required for subsequent post-translational modifications (e.g., phosphorylation and subsequent binding of 14-3-3 protein). Thus, only a very small portion of AHA2 protein is phosphorylated, but the phosphorylation status changes significantly in activated class C mutants. In contrast, the phosphorylation status of AHA2 and its activated mutants is very similar (16).

Upon arrival at the plasma membrane, many transporters undergo phosphorylation (24, 25). In several documented cases, phosphorylation occurs on serine residues, and some of the required kinases have been identified (26, 27). In a number of cases, this post-translational modification is required for a subsequent modification, ubiquitination, that occurs at the plasma membrane (26, 28, 29). In this case, post-translational modification is a signal required for endocytosis. We have demonstrated yet another example of post-translational modification at the plasma membrane, which leads to the binding of regulatory 14-3-3 protein. As 14-3-3 proteins bind to a large number of eukaryotic target proteins including membrane-bound transporters, the ability of endogenous protein kinase(s) to recognize the 14-3-3 binding motifs might govern the activity of these proteins not only in heterologous systems such as yeast but also in their natural hosts.

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