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Genetic Analysis of Regulatory Mutants Affecting Synthesis of Extracellular Proteinases in the Yeast *Yarrowia lipolytica*: Identification of a *RIM101/pacC* Homolog

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Depending on the pH of the growth medium, the yeast *Yarrowia lipolytica* secretes both an acidic proteinase and an alkaline proteinase, the synthesis of which is also controlled by carbon, nitrogen, and sulfur availability, as well as by the presence of extracellular proteins. Recessive mutations at four unlinked loci, named *PAL1* to *PAL4*, were isolated which prevent alkaline proteinase derepression under conditions of carbon and nitrogen limitation at pH 6.8. These mutations markedly affect mating and sporulation. A dominant suppressor of all four *PAL* mutations was isolated from a wild-type genomic library, which turned out to be a C-terminally truncated form of a 585-residue transcriptional factor of the His_2Cys_2 zinc finger family, which we propose to call YlRim101p. Another C-terminally truncated version of YlRim101p (419 residues) is encoded by the dominant *RPH2* mutation previously isolated as expressing alkaline protease independently of the pH. YlRim101p is homologous to the transcriptional activators Rim101p of *Saccharomyces cerevisiae*, required for entry into meiosis, and PacC of *Aspergillus nidulans* and *Penicillium chrysogenum*, which were recently shown to mediate regulation by ambient pH. YlRim101p appears essential for mating and sporulation and for alkaline proteinase derepression. *YlRIM101* expression is autoregulated, maximal at alkaline pH, and strongly impaired by *PAL* mutations.

Secretion of aspecific proteolytic activities is a widespread characteristic of many yeast species. In a recent literature survey of 110 yeast species representing 31 genera, around 80% were found to secrete proteinases (37). Surprisingly, little is known about the regulation of these extracellular proteinases, although it is widely admitted that they may significantly contribute to yeast ecological distribution and potential pathogenicity and play a major role in biotechnological applications. Purification, characterization, and regulation of proteinases have been reported for seven species (37), and since *Saccharomyces cerevisiae* does not secrete any proteinase, most studies have focused on two species, *Candida albicans*, which secretes at least seven acidic aspartyl proteinases (Sap) possibly involved in pathogenicity (10, 32), and *Yarrowia lipolytica*, which secretes both an acidic aspartyl proteinase (AXP) and an alkaline seryl proteinase (AEP), depending on the pH of the growth medium (1). AEP secretion and processing have been extensively studied (14, 15, 30, 31), and AEP was used as a model reporter molecule to investigate protein secretion in this yeast (6, 20, 52). The regulation of *C. albicans* and *Y. lipolytica* extracellular proteinases appears to be quite complex, but the mechanisms involved have some elements in common. Their synthesis is strongly repressed by low-molecular-weight nitrogenous substrates (ammonia, glutamate, and urea) and mildly sensitive to repression by preferred carbon sources such as glycerol or glucose, whereas they are strongly induced in media containing high-molecular-weight proteinaceous substrates (22, 34, 35, 41, 51). Besides carbon-nitrogen availability and the presence of inducing proteins, the pH of the growth medium appears to be a major determinant. It has been questioned whether pH is a primary regulatory determinant or if it acts indirectly, by modulating the accessibility of proteolytic peptides, which may be the actual inducers (22). As a rule, however, acidic proteinases progressively disappear when the pH is raised from 3.2 to neutral (18, 34) and are replaced by AEP in *Y. lipolytica* or by specific Sap isoenzymes in *C. albicans* (50). Interestingly, very similar induction conditions have been reported for extracellular neutral and alkaline proteinases of *Aspergillus nidulans* (8, 24) and may generally apply to many extracellular fungal proteinases. In all of the cases investigated, regulation of proteinase synthesis has been shown to occur mainly at the transcriptional level.

Conservation at the molecular level, if any, of the underlying regulatory mechanisms is totally unknown, and the nature of the peptone-derived inducing factor(s) remains elusive (50). By using the genetically tractable yeast *Y. lipolytica* (3) as a model organism, we started a molecular analysis of the regulation of the *XPR2* gene, which encodes the strongly expressed AEP (11, 45). By combining deletion analysis of the 800-bp-long *XPR2* promoter, site-directed mutagenesis, and in vivo protein footprinting, we previously identified two major upstream activating sequences (UAS) required for its transcriptional activation (5). Deletion analysis of either UAS drastically reduced *XPR2* promoter efficiency but failed to identify a specific target for carbon and nitrogen response or for peptone inducibility. In view of the complexity of the *XPR2* promoter and of its regulation, we decided to isolate *trans*-acting regulatory mutants.

This paper reports on the genetic and physiological characterization of mutations, affecting four unlinked genes, which abolish the transcriptional activation of the *XPR2* promoter. We further report on the cloning of a suppressor of these mutations, which identified a homolog of the transcriptional activators Rim101p (formerly called Rim1p) of *S. cerevisiae*,

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required for entry into meiosis (46), and PacC of *A. nidulans* (38, 49), *A. niger* (26), or *Penicillium chrysogenum* (47), which has been recently shown to mediate regulation by ambient pH.

MATERIALS AND METHODS

Strains. The bacterial strains used for transformation and amplification of recombinant DNA were *Escherichia coli* HB101 and DH5α (42). Isogenic strains of *Y. lipolytica* AM3 and AM4 (9) carry nonreverting *ura3* and *leu2* mutations, an *XPR2-lacZ* fusion integrated at the *XPR2* locus (Fig. 1), and an *XPR2-SUC2* fusion disrupting the *URA3* gene (*ura3-302* allele) which directs the expression of the *S. cerevisiae* invertase (3). When needed, the *LEU2* marker of strain AM3 or AM4 was disrupted with the *URA3* marker to generate strains LAM57 and LAM58, respectively (Fig. 1). Similarly, we disrupted the *LEU2* gene in the Pal mutants (see Results). When needed, secondary Ura$^-$ clones, resulting from rare pop-out events that left the *XPR2-lacZ* fusion, were selected on 5-fluoroortic acid medium. See Table 2 for details.

Culture media and phenotypic tests. Complete YPD and minimal YNB-glucose medium have been described by Sherman et al. (44). The derepressing medium (Y) contained 1% yeast extract. The protease-inducing (YPDm) medium has been described previously (5). Solid and liquid media were buffered either at pH 4.0 (with 0.2 M citrate buffer) or at pH 6.8 (with 0.2 M phosphate buffer) unless otherwise indicated. The Lac$^+$ phenotype was screened on Y medium supplemented with 70 mg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) per ml and buffered at pH 6.8 (Y-XGal$^{6.8}$). For β-galactosidase assays, culture samples were centrifuged at 5,000 rpm for 5 min in a Biofuge 1S (Heraeus Instruments GmbH, Manau, Germany), washed once with 0.1 M phosphate buffer (pH 7.0), resuspended in this buffer to reconstitute the volume of the original samples, and then assayed as described previously (5), on two to four independent cultures of the same strain. AEP activity (Aep$^+$ phenotype) was screened on colonies spotted on skim milk plates (36). The sucrose-utilizing phenotype (Suc$^+$) was tested on YNB$^{6.8}$ medium with glutamate as a nitrogen source and filter-sterilized sucrose as a carbon source. Growth rates at various pHs were compared in YPD medium containing 0.2 M citric acid-phosphate buffer at pH 3.5 or phosphate buffer at pH 6.8 or 8.0. At the end of the culture period, pHs were about 4.1, 6.9, and 7.9, respectively.

Mating and sporulation assays. Compatible strains carrying complementary auxotrophic markers were derived from *PAL* or *YlRIM101* mutated strains by crossing with their isogenic AM3 and AM4 parents or by transformation. Diploids were selected on minimal medium by cross replica plating of compatible auxotrophs (3). Mating efficiency was evaluated after 3, 4, and 5 days at 28°C as the number of clones formed at fertile intersections. Since some *Y. lipolytica* diploids tend to be unstable (3), sporulation was best tested by plating a pool of diploids on CSM (3) and checking ascus formation under a microscope after 3, 4, and 7 days at 25°C. Some diploids were obtained from a parent(s) transformed with a replicating plasmid. Curing of the plasmid was obtained by passaging diploids on YPD and checking loss of plasmid-associated phenotypes. Several clones were then tested for sporulation. Sporulation was scored as positive when both sporulating and nonsporulating clones were obtained for a given genotype and negative when none of the tested diploids sporulated.

Construction of strains with *YlRIM101* deleted. A first deletion (*Ylrim101-1018*) removing the zinc finger (Zf) region and replacing it with the *LEU2* marker was constructed as follows. The Zf coding region (positions 157 to 914; see Fig. 4) was removed from an *Exo*III derivative of pINA1014 (Fig. 2) after digestion by *Nhe*I and *Nsi*I and replaced with an *Nhe*I-*Pst*I fragment of pINA240 (3) carrying the *LEU2* gene. The resulting plasmid, pINA1018, was digested by *Sph*I and *Nco*I, and the fragment carrying the disrupted *YlRIM101* sequence was integrated by double crossover at the *YlRIM101* locus into strains LAM57 and LAM58.

A complete deletion (*Ylrim101-1113*) was constructed by using a PCR strategy (Fig. 1B) adapted from that of Maftahi et al. (29). The 1,715-bp PCR2 fragment was digested by *Hin*dIII and *Bam*HI and then inserted into pINA300′ (carrying *URA3*; see reference 3), which was digested by the same enzymes to generate plasmid pINA1113. A two-step gene replacement technique was used to replace the resident *YlRIM101*$^+$ allele of AM3 or AM4 with *Ylrim101-1113*. First, *Spe*I-digested pINA1113 was targeted to the terminator region of *YlRIM101*, selecting for Ura$^+$ clones. Ura$^-$ clones were then selected on 5-fluoroorotic acid medium, and pop-out events leaving the *Ylrim101-1113* deletion (strains FL3ΔR and SBR109) were selected.

Construction of strains carrying truncated *YlRIM101* alleles. The *Hin*dIII-*Bam*HI fragment from pINA1014 (*YlRIM101-937* allele, Fig. 2A) was cloned into pINA62 (3), which was cleaved by the same enzymes to generate pINA1112. Integration of this plasmid following *Not*I digestion was targeted downstream from the *LEU2* locus of LAM57 or Pal mutants (Table 1). A second plasmid, in which the last codon of this truncated *YlRim101* gene was abutted to the natural *YlRIM101* terminator (pINA1116 in Fig. 2C), was constructed as follows. The 3,746-bp *Hin*dIII-*Nae*I fragment from pINA1014 (Fig. 2A) and the 291-bp *Stu*I-*Spe*I fragment from pINA1019 (Fig. 2B) were recombined into *Hin*dIII- and *Nhe*I-cleaved pINA1115, a pINA300′ (3) derivative carrying a unique *Not*I site within the pBR322 part. This plasmid was targeted by *Not*I digestion to the pBR322 sequence present at the *XPR2* locus (Fig. 1) of strain FL3ΔR to generate FL4RΔC (*YlRIM101-1116* allele).

We previously identified the *RPH2* locus, where dominant mutations conferred on AM3 a pH-independent phenotype for the expression of alkaline protease (9). Conventional genetic data suggested that *RPH2* and *YlRIM101* might be allelic. A nonsense mutation (CAG to TAG) was identified by direct sequencing of PCR products derived from the *YlRIM101* locus of the mutant PH5 (9). This allele (now *YlRIM101-5*) truncates YlRim101p at position 419.

A third C-terminal deletion of *YlRIM101* mimicking the putative in vivo truncation was constructed by abutting codon 330 to the natural *YlRIM101* terminator. A PCR strategy similar to that described in Fig. 1B was used. The region upstream from residue 330 was amplified with oligonucleotides 1 (5′-CACCTACGCAAGTGGCTACG) and 2 (5′-AGGTTCTTTATCGGGCTCGCTTGATGTCG) on plasmid pINA1014 (Fig. 2A); the region encompassing the stop codon and the terminator was amplified by using oligonucleotides 3 (5′-GCGAGCCCGATAAAGAACCTTCTGGAATTCGG) and 4 (5′-CAGACTTCCCGGGTTTCCTC) on plasmid pINA1019 (Fig. 2B). The resulting PCR2 product was digested by *Nsi*I and *Dra*III and then inserted between *YlRIM101* flanking sequences (plasmid pINA1119). The DNA sequence of the truncation was checked. *Dra*III-digested pINA1119 was targeted to the terminator of *YlRIM101* in strains AM3 and AM4, selecting for Ura$^+$ clones. Secondary Ura$^-$ clones were selected on 5-fluoroorotic acid medium, and recombination events leaving the *YlRIM101-1119* truncation (strains AM319ΔC and AM419ΔC) were selected.

RNA analysis. Cells were grown at 23°C in YPDm$^{4.5}$ or YPDm$^{6.8}$ and harvested in the late logarithmic phase (optical density at 600 nm, about 4). pH values at harvesting were within 0.4 U of the initial values. Total RNA was extracted from about 1 g of centrifuged wet cells with a kit from Promega Corporation (Madison, Wis.), with the following modifications. After one wash with cold diethyl pyrocarbonate-treated water, the pellet was resuspended in 4 ml of the denaturing solution from the kit and 4 g of baked, acid-washed 0.45-mm glass beads (Sigma) was added. The whole was mixed vigorously on a Vortex mixer for 7 × 30 s with intermittent cooling on ice for 30 s. As described in reference 42, electrophoresis of 40 μg of each total RNA was carried out on a 6.6% formaldehyde–1.2% agarose gel with an RNA ladder (0.24 to 9.5 kb; Bethesda Research Laboratories) as size standards. Northern (RNA) analysis was performed as previously described (16). Hybridized membranes were autoradiographed and analyzed by using an actin transcript as an internal standard. All Northern data were confirmed with at least two independent RNA preparations.

DNA techniques. Yeast total DNA was extracted by the method of Hoffman and Winston (21). Standard recombinant DNA techniques were performed essentially as previously described (42). *E. coli* was transformed as described by Hanahan (19). Transformation of *Y. lipolytica* was done as previously described (3). All transformation events were confirmed by Southern analysis and, in some cases, by PCR. The 3′ end of the *YlRIM101* gene was recloned by amplification of genomic DNA. Total DNA of a wild-type strain was digested by *Nhe*I, recircularized in vitro, and digested by *Aat*II (Fig. 2F). Amplification using oligonucleotides 13 (at position +214 [see the sequence in Fig. 4]; 5′-CGCCCACATTCATGTTG) and 10 (at position +725; 5′-ACAACGCTTACGCCAAG) was performed with 40 cycles of PCR as recommended by the supplier of *Taq* DNA polymerase (Appligène). From the amplified 3.3-kb fragment, a 1.8-kb *Bal*I fragment was isolated by partial restriction and cloned into pBSKS$^-$ (Stratagene) to generate plasmid pINA1019 (Fig. 2D), which was used for sequencing. The sequence of the 3′ end of the *YlRIM101* open reading frame (ORF) was further confirmed by direct sequencing of the amplified PCR products. DNA sequencing and sequence analysis were performed as described by Maftahi et al. (28).

Nucleotide sequence accession number. The sequence of *YlRIM101* has been deposited in the EMBL nucleotide sequence database under accession number X99616.

RESULTS

Mutant isolation. Our aim was to identify genetic loci involved either positively or negatively in the control of the *XPR2* promoter. We thus set out to isolate *trans*-acting mutations which either prevent *XPR2*-driven expression on derepressing medium (Y) or, on the contrary, force expression under conditions in which the promoter is usually silent (YNB). To eliminate *cis*-acting mutations or mutations acting on downstream steps of alkaline protease (AEP) expression (maturation or secretion), we constructed first a set of isogenic strains coexpressing AEP and a reporter activity (β-galactosidase), both under the control of the wild-type *XPR2* promoter. The two compatible, isogenic strains AM3 and AM4 contain an *XPR2-lacZ* transcriptional fusion integrated at the *XPR2* locus on chromosome V (Fig. 1). Both strains score Lac$^+$ and Aep$^+$ (i.e., they turn blue on Y–X-Gal and form halos on skim milk

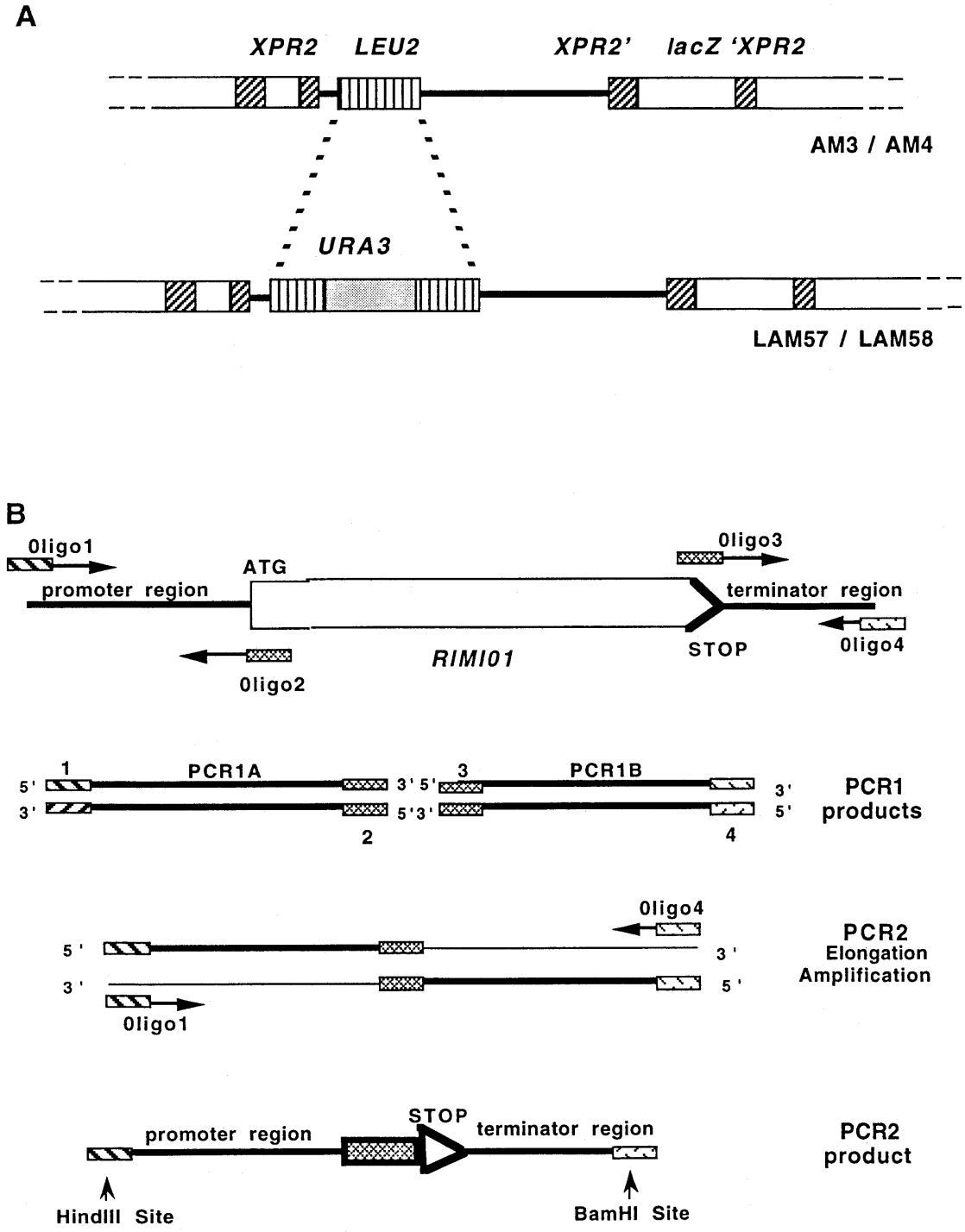

FIG. 1. (A) Schematic representation of the *XPR2* locus in strains AM3 and AM4 and strains LAM57 and LAM58. pINA404 (5) was targeted by *Kpn*I digestion to the *XPR2* terminator sequence of strains E122 and 22301-3 (3) to generate strains AM3 and AM4, respectively. PBR322 sequences are indicated by thick lines, and *XPR2* promoter and terminator sequences are represented as boxes with oblique lines. Strains LAM57 and LAM58 were obtained from AM3 and AM4, respectively, by disrupting the *LEU2* gene by double crossover using a construct in which the 29-bp *Xho*I fragment within the *LEU2* ORF had been replaced with a 1,684-bp *Sal*I fragment of pINA300′ (3) carrying *URA3*. (B) Schematic view of the PCR-based strategy used to create the *YlRIM101* complete deletion. A first PCR was performed to amplify the promoter and terminator regions separately. For promoter amplification (PCR1A product), oligonucleotides (oligo) 1 (5′-TGCC**AAGCTT**AGTGTA CGATACTGAGAGG) and 2 (5′-CCCCGGCGCGCCCCCCCTGTCGTTACCTCACTAAAC) were used on pINA1014 (see Fig. 2A). For terminator amplification (PCR1B product), oligonucleotides 3 (5′-GGGGGGCGCGCCGGGGTGCTAAAGAACCTTCTGG) and 4 (5′-AGGG**GGATCC**GTAACTCTCAACAGGTGATG) were used with pINA1019 (Fig. 2B). Oligonucleotides 1 and 4 each contain an additional sequence (boldface) carrying one restriction site (*Hin*dIII and *Bam*HI, respectively). Oligonucleotides 2 and 3 contain 16 nucleotides of the complementary sequence (hatched boxes; underlined in the above sequences). The PCR1A and PCR1B products were annealed through their sticky ends and further amplified by using oligonucleotides 1 and 4 as described by Maftahi et al. (29). The resulting PCR2 product was digested by *Hin*dIII and *Bam*HI to construct pINA1113 (see Materials and Methods).

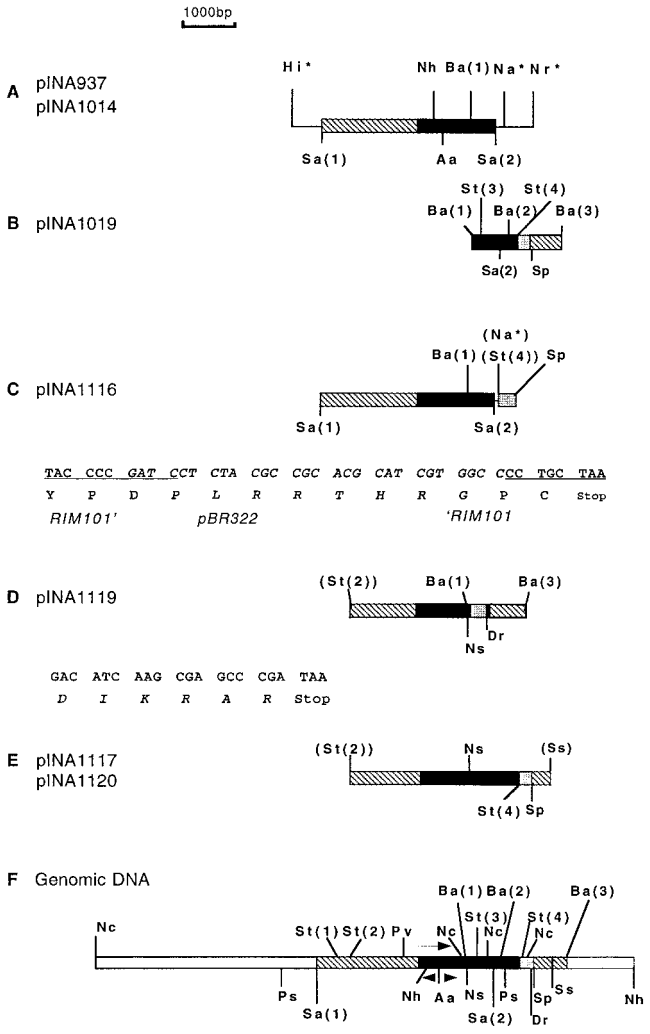

FIG. 2. Restrictions maps of plasmid inserts and genomic DNA carrying *YlRIM101*. (A) The *pal* suppressor fragment, containing the carboxy-terminally truncated *YlRIM101-937* allele, is carried in vectors pINA240 (3) and pBSKS$^-$ for pINA937 and pINA1014, respectively. The solid black box indicates the *YlRIM101* ORF, the striped box represents sequenced flanking DNA, and the thin black line represents pBR322 DNA. (B) The insert containing the 3′ end of the *YlRIM101* ORF and its flanking DNA is carried by pBSKS$^-$. The shaded region indicates the putative *YlRIM101* transcription terminator. (C and D) Schematic representations and amino acid sequences of truncated versions of *YlRIM101-1116* (C) and *YlRIM101-1119* (D), both abutted to the *YlRIM101* transcription terminator. (E) The insert containing the whole *YlRIM101* ORF and its flanking DNA as carried in integrative and replicative vectors pINA1117 and pINA1120, respectively. (F) Restriction map of the genomic region encompassing *YlRIM101*. Open bars represent flanking regions not sequenced. The direction of transcription is indicated by the arrow. Arrowheads upstream and downstream from the *Aat*II site stand for oligonucleotides 13 and 10, respectively (see Materials and Methods). Abbreviations: Aa, *Aat*II; Ba, *Bal*I; Bc, *Bcl*I; Dr, *Dra*III; Hi, *Hin*dIII; Na, *Nae*I; Nc, *Nco*I; Nh, *Nhe*I; Nr, *Nru*I; Ns, *Nsi*I; Ps, *Pst*I; Pv, *Pvu*I; Sa, *Sau*3A; Sp, *Spe*I; Ss, *Sst*I; St, *Stu*I. Asterisks indicate sites in flanking vector sequences. Sites that were destroyed during construction are in parentheses. Repeated sites are identified by numbers.

plates) when grown on Y medium and Lac$^-$ and Aep$^-$ when grown on YNB medium (both at pH 6.8). Mutants simultaneously altered in the expression of both activities were isolated in two independent UV mutageneses at survival rates of 43.5 and 32.5% in the case of AM3 or 24 and 18% in the case of AM4. All mutants saved after two rounds of purification were backcrossed two or three times to AM3 or AM4. Two types of mutants were sought.

Constitutive mutants were isolated as blue colonies on YNB–X-Gal[6.8] and further checked for AEP activity. Clones scoring positive in both tests appeared at a frequency of 10^{-4} per surviving cell. Most of these mutants crossed poorly and/or were meiotically unstable. None could be conserved beyond the third backcross to AM3 or AM4, so this class was not studied further.

Nonderepressible mutants were isolated as Lac$^-$ and Aep$^-$ when grown on Y[6.8] medium. They appeared at a rather high frequency ($2 \cdot 10^{-4}$ to 10^{-3} per surviving cell), but many clones turned out to be unstable and were lost during purification and/or backcrossing to AM3 or AM4. Of 40 mutants each isolated from AM3 and AM4, 7 monogenic mutants were finally saved and called Pal21 to Pal27 in deference to the better-studied *pal* genes of *A. nidulans* (see Discussion and reference 2). They all carried recessive mutations which were assigned to four complementation groups by testing of all pairwise diploid combinations: *pal1-22*, *-25*, and *-26*; *pal2-23* and *-27*; *pal3-21*; and *pal4-24*. Since each complementation group was defined by only one to three alleles, several more genes are likely to be involved in the activation of the *XPR2* promoter.

Phenotype of Pal mutants. To confirm that the different mutations were indeed *trans*-acting, we took advantage of the presence in all Pal strains of an *XPR2-SUC2* fusion which disrupts the *URA3* locus on chromosome IV (*ura3-302* allele) and confers a sucrose utilizing phenotype on these strains upon *XPR2* induction (3). In contrast to AM3 and AM4, all Pal mutants scored Suc$^-$.

To test whether the Pal mutants remained sensitive to induction by peptones, β-galactosidase activity was measured in cell extracts of one strain of each complementation group grown on YPDm[6.8] medium. No activity was detected, indicating that Pal mutants could not be induced by peptones.

To detect a possible pH-dependent phenotype, we quantitated the level of expression of extracellular proteinase genes at both acidic and alkaline pHs. To quantitate *XPR2* expression, β-galactosidase activity was measured in cell extracts of Pal22, Pal23, Pal21, and Pal24 mutants grown on YPDm medium at either pH 4.0 or 6.8. The results are shown in Table 1; in all four types of Pal mutants, β-galactosidase levels remained very low at both pHs. This was subsequently confirmed by Northern analysis (Fig. 3A) showing that *XPR2* transcripts remained undetectable in the mutants under all pH conditions. A similar analysis was performed to monitor the transcription levels of *AXP*, the structural gene of the acidic proteinase (Fig. 3A). In the control AM3 strain, *AXP* transcript levels were low at pH 6.8 and high at pH 4.5. This pattern was significantly affected in Pal mutants. At acidic pH, *AXP* transcription was significantly decreased, except in the Pal22 (*pal1-22*) strain, in which marked derepression of *AXP* transcripts was observed. Three Pal mutants still failed to transcribe *AXP* at alkaline pH, but moderate expression was reproducibly detected in the Pal23 (*pal2-23*) mutant.

Isolation of plasmids complementing the *PAL* defects. To isolate genes complementing the *PAL* defects, we used a *Y. lipolytica* library made in the *LEU2 ARS18* replicative, centromeric vector pINA240 (3, 17). The library consists of five pools of 4,200 clones (80% hybrids) containing *Sau*3A inserts of wild-type genomic DNA with a mean size of 3.5 kb (3).

The *LEU2* gene integrated at the *XPR2* locus of the seven Pal mutants was disrupted by a *URA3* cassette so as to restore a Leu$^-$ phenotype (Fig. 1). The resulting strains were tested for high efficiency of transformation (more than $4 \cdot 10^5$ transformants per μg of pINA240 DNA). Three strains carrying the

TABLE 1. Effects of *PAL* and *YlRIM101* mutations on *XPR2-lacZ* expression

Ambient pH	Presence of pINA1112 containing *YlRIM101-937*[b]	β-Galactosidase activity[a]						
		LAM57 (wild type)	Pal22 (*pal1-22*)	Pal23 (*pal2-23*)	Pal21 (*pal3-21*)	Pal24 (*pal4-24*)	PH5 (*YlRIM101-5*)	AM319ΔC (*YlRIM101-1119*)
6.8	−	229	0.7	1.3	1.5	1	225	76
	+	249	281	338	277	275		
4.0	−	7.6	<0.1	<0.1	0.1	<0.1	139	69
	+	6.9	10.5	15.9	14.8	17.4		

[a] β-Galactosidase activities were assayed in stationary-phase cultures in YPDm medium buffered at the indicated pH. Values are given in Miller units and normalized for the optical density at 600 nm of the culture broth.
[b] When indicated (+), one copy of the (3′ truncated) *YlRIM101-937* allele was integrated at the *LEU2* locus of the corresponding strain.

pal1-22, *pal2-23*, and *pal3-21* mutations were transformed with the library, and transformants were selected on leucine-free glucose or sucrose minimal medium. All transformants were subsequently tested for restoration of the Lac^+ phenotype on Y–X-Gal[6.8]. About two to four Lac^+ Suc^+ clones were retained for each Pal strain transformed.

To eliminate clones resulting from a chromosomal suppressive event, all candidates were serially passaged onto YPD medium to permit segregation of the transforming plasmid(s). All of the Leu^- clones observed scored Lac^- and Suc^- simultaneously, confirming that restoration of the *XPR2* promoter activity was plasmid linked. Finally, the plasmids present in the transformants were rescued by transformation of *E. coli* HB101 and then back transformed into each Pal mutant. As expected, they conferred the Lac^+ and Suc^+ phenotypes in each case.

These plasmids were characterized by restriction analysis. Only two kinds of plasmids were rescued from the transformants. Plasmid pINA935, carrying a 6-kb insert, was rescued from two of the four *pal2* transformants tested. This plasmid complemented both the *pal2-23* and *pal2-27* alleles but none of the *pal1*, *pal3*, and *pal4* alleles tested (data not shown). Plasmid pINA937, carrying a 3.4-kb insert (Fig. 2A), was rescued from *pal1*, *pal2*, and *pal3* transformants. This plasmid was subsequently shown to restore *XPR2* activity to all Pal mutants, including the Pal24 (*pal4-24*) mutant, and thus carried a suppressor common to all *pal* mutations.

To check whether the 3.4-kb insert is able to suppress each *pal* mutation when integrated as a single copy in the genome, the fragment was recloned in the integrative vector pINA1112. A single copy of this construct was targeted to the *LEU2* locus of AM3 and of each Pal mutant. β-Galactosidase activity was measured on cell extracts of the resulting strains grown on YPDm[6.8] medium. Results clearly indicated that a single copy of the 3.4-kb insert was able to efficiently suppress each *pal* mutation at pH 6.8 (Table 1).

Sequence analysis of a *RlM101*/*pacC* homolog in *Y. lipolytica*. The insert of plasmid pINA937 was sequenced by using a combination of subcloned fragments, *Exo*III deletions, and primer walking. The resulting sequence (Fig. 4) evidenced a large, 473-amino-acid reading frame which had been fortuitously truncated at a *Sau*3A site at its 3′ end during the construction of the genomic library. This truncation resulted in a translational fusion of this ORF with a downstream plasmid-borne ORF. A PCR strategy was devised for recloning of the missing 3′ end of the gene (see Materials and Methods and Fig. 2B and F).

The complete nucleotide and amino acid sequences of the reconstituted gene are shown in Fig. 4. A first homology search conducted on the predicted protein sequence (585 residues) identified two transcriptional regulators: Rim101p (formerly called Rim1p [46]) of *S. cerevisiae* (27.6% identity) and PacC (49) of *A. nidulans* (34.6% identity). Since then, two additional PacC homologs were identified in *A. niger* (26) and in *P. chrysogenum* (47). Like its homologs, YlRim101p carries three Zfs of the Cys_2His_2 class, numbered Yl-1, Yl-2, and Yl-3, which can be aligned with the Zf consensus proposed by Jacobs (23) (see Fig. 5). Sequence conservation among the five homologs is very strong for Zf2 and Zf3 and weaker for Zf1. In PacC proteins, as in GLI (40), this finger is not thought to contact the DNA. It should be noted that, like Zf1 of its Rim101/PacC homologs, Yl-1 carries the Trp residue located in the Cys knuckle (position c3), which might establish a hydrophobic interaction with another Trp residue in the equivalent position of Zf2; such an interaction in GLI (40) makes critical hydrophobic contacts at the interface of Zf1 and Zf2. The Zf2 linker is perfectly conserved among the five proteins. Like Zf1 of tra-1 (54) and other Rim101/PacC proteins, Yl-1 has an unusually long linker (one turn of the DNA helix instead of the half turn for the consensus cited just above). Despite these features, the primary structure of Yl-1 is more similar to the consensus of Jacobs (23) than are the Zf1s of the Rim101/PacC homologs (Fig. 5). Indeed, Yl-1 carries the strongly conserved basic Lys residue at position r3, which contacts the sugar phosphate backbone of DNA (39, 40), and presents residues at positions s3, s6, and m3 which directly contact bases in the Zif268-DNA and GLI-DNA complexes (39, 40). At the f1, s1, and m1 hydrophobic core positions known to be necessary to create a fold in the structure with the zinc ligands, both Yl-1 and Yl-3 possess the conserved s1 Phe and m1 Leu residues; this last residue is conservatively replaced by Ile in Yl-2. At position r2, both Yl-1 and Yl-2 carry a strongly conserved Gly residue which is most variable in homologous fungal fingers (23). Since the binding site of YlRim101p remains to be determined (see Discussion), these data suggest but do not prove that Yl-1 may contact DNA.

No convincing conservation was found outside the Zf region when the five proteins were aligned. In common with *A. nidulans* PacC and other transcription factors, YlRim101p carries several S/TPXX motifs, six SPXX and seven TPXX (48). Unlike its PacC homologs, the N-terminal region, upstream from the Zfs, is not Ala rich but carries a long stretch of Gln residues (three smaller stretches are present downstream from the Zfs). Within the central region, like its two *Aspergillus* PacC homologs, YlRim101p carries a Tyr-rich region and three Pro-Gly-rich regions, but no Ser-Thr-rich region. As in the Rim101/PacC homologs, the C-terminal region is quite acidic: 17 acidic residues are found between positions 509 and 570. Although this region is required for full transcriptional activity in *S. cerevisiae* (46), its role is likely to be different in filamentous fungi and *Y. lipolytica* (see Discussion).

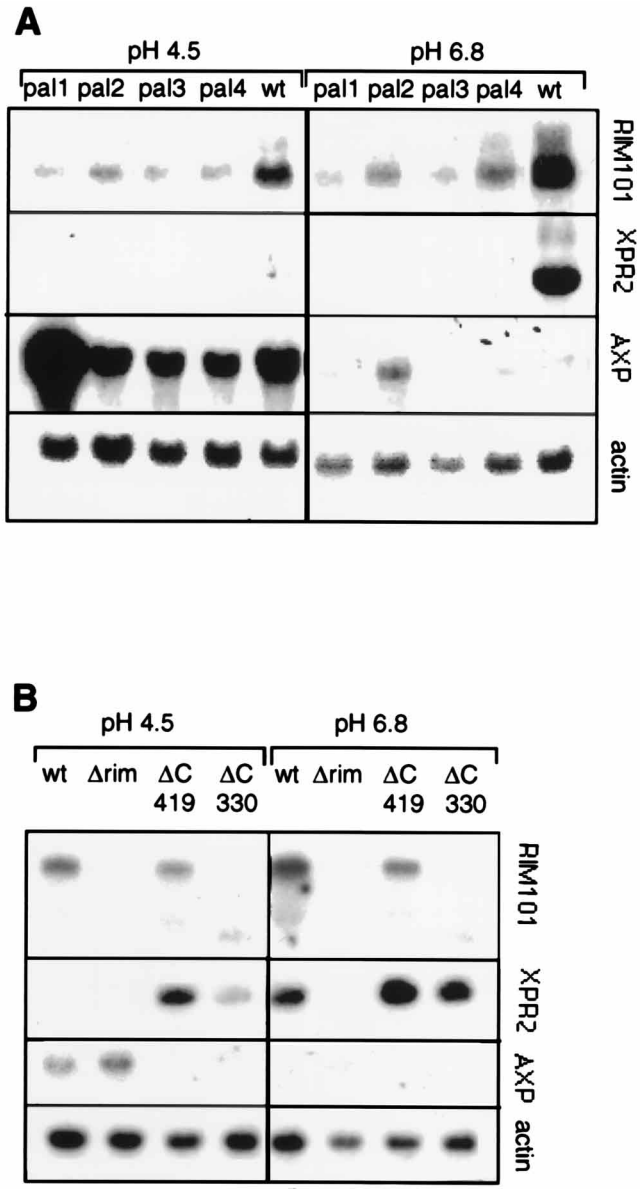

FIG. 3. Effects of ambient pH and of *PAL* or *YlRIM101* mutations on *XPR2*, *AXP*, and *YlRIM101* transcript levels. Strains were grown in YPDm medium at the indicated pHs, and RNAs were extracted. About 35 μg of total RNA was electrophoresed in each case and analyzed by Northern blotting. The probes used are indicated on the right. The *YlRIM101* probe was a 0.92-kb *Nhe*I-*Stu*I fragment from pINA1014 (nucleotides 158 to 1075; see Fig. 4); the alkaline proteinase gene (*XPR2*) probe was a 0.88-kb *Bgl*II fragment from pINA344 (5) included in the *XPR2* coding sequence; the acid proteinase gene (*AXP*) probe was a 0.26-kb *Bam*HI-*Nco*I fragment, kindly given by D. Glover, completely included in the coding sequence; and the actin probe was a 1.07-kb *Sca*I-*Xho*I fragment from pINA1101 containing the *Y. lipolytica ACTI* gene (5a). (A) Northern blots of RNAs extracted from strains carrying the mutation *pal1-22* (pal1), *pal2-23* (pal2), *pal3-21* (pal3), or *pal4-24* (pal4) and wild-type (wt) strain AM3. (B) Northern blots of RNAs extracted from strains AM3 (wt), FL3ΔR (Δrim), PH5 (ΔC419), and AM319ΔC (ΔC330).

Since PacC has been shown to undergo pH-dependent truncation in vivo (38), we tried to identify a possible proteolytic site in YlRim101p. The amino acid sequences of PacC and YlRim101p were aligned and compared by using the hydrophobic cluster analysis method (25). A region around residue 330 of YlRim101p showed a profile similar to that of the expected proteolytic site of PacC (data not shown) and was located at the same distance from the Zfs as the predicted site in PacC. As shown below, a YlRim101p version truncated at this position is transcriptionally active and renders *XPR2* transcription unresponsive to ambient pH.

***YlRIM101* identifies a gene different from *PAL1*, *PAL2*, *PAL3*, and *PAL4*.** To create a *YlRIM101* negative mutant and to tag the *YlRIM101* locus, *YlRIM101* was disrupted in haploid strains LAM57 and LAM58 by using plasmid pINA1018 (see Materials and Methods). This replaced the Zf region (positions 157 to 914 of the coding sequence) with a *LEU2* marker. All disrupted strains scored Lac^-, suggesting that, like *pal* mutations, *Ylrim101-1018* abolishes *XPR2* transcription.

Ylrim101-1018 disruptants were crossed to strain AM3 or AM4. The resulting diploids exhibited a Lac^+ phenotype, showing, as expected, that *Ylrim101-1018* is recessive. Further genetic analysis showed that *YlRIM101* defines a new gene distinct from *PAL1* to *PAL4*. *YlRIM101* was assigned to chromosome II of strain E150 after hybridization to pulsed-field gel electrophoresis-separated chromosomes (data not shown).

***YlRIM101* is pH regulated and required for derepression of alkaline proteinase.** To definitely establish the phenotype of a *YlRIM101* null mutation, strain FL3ΔR carrying the *Ylrim101-1113* allele was constructed (see Materials and Methods). The deletion starts 26 bp upstream from the putative initiator codon and terminates 4 bp upstream from the stop codon. The transcriptional activity of the *XPR2* promoter was assessed by measuring β-galactosidase activities in strains AM3 and FL3ΔR on $YPDm^{4.0}$ or $YPDm^{6.8}$. The null strain exhibited only 1% of the activity of the control at pH 6.8 and no activity at pH 4.0. This shows that YlRim101p is absolutely required for transcriptional activation of *XPR2* at pH 6.8 but not for its shutdown at pH 4.0. This was confirmed by Northern blot analysis of total RNA extracted from late-log-phase cells (Fig. 3B).

To test the effects of pH and of *pal* mutations on *YlRIM101* expression, total RNA was probed for *YlRIM101* mRNA. In control strain AM3, a single 1.8-kb *YlRIM101* transcript was detected on $YPDm^{6.8}$, the level of which was reduced by about 50% at acidic pH (Fig. 3A and B). In the Pal mutants, *YlRIM101* mRNA was markedly reduced at both acidic and alkaline pHs. Strong suppressive alleles of *YlRIM101*, like *YlRIM101-5* or *-1119* (Table 1), resulted in pH-independent expression of *YlRIM101* (Fig. 3B).

To assess the effect, if any, of *Ylrim101-1113* on the expression of the acidic extracellular protease, Northern analysis was conducted as described above to probe *AXP* mRNA (Fig. 3B). In the null strain, *AXP* transcript levels were not significantly affected at acidic pH and remained undetectable at pH 6.8. Thus, YlRim101p is required neither for *AXP* expression at acidic pH nor for its shutdown at alkaline pH.

Transcriptional effects of carboxy-terminal truncations of *YlRIM101*. The truncated allele *YLRIM101-937*, initially isolated as a dominant suppressor of *pal* mutations in a $YLRIM101^+$ context, carried a translational fusion of *YlRIM101* at amino acid 473 with a downstream plasmid-borne ORF. To establish the phenotype of a strain expressing only the truncated YlRim101p polypeptide, we inserted a stop codon and a transcriptional terminator at the fusion joint (*YlRIM101-1116* allele) and used this construct to replace the wild-type *YlRIM101* gene in strain FL4RΔC (see Materials and Methods and Fig. 2C). Northern analysis (data not shown) showed that the *YlRIM101* transcript was barely detectable in this strain at both acidic and alkaline pHs. *XPR2* transcription, however, appeared fully restored at pH 6.8 in Pal mutants but was only slightly derepressed at pH 4.5 (see also Table 1). This sug-

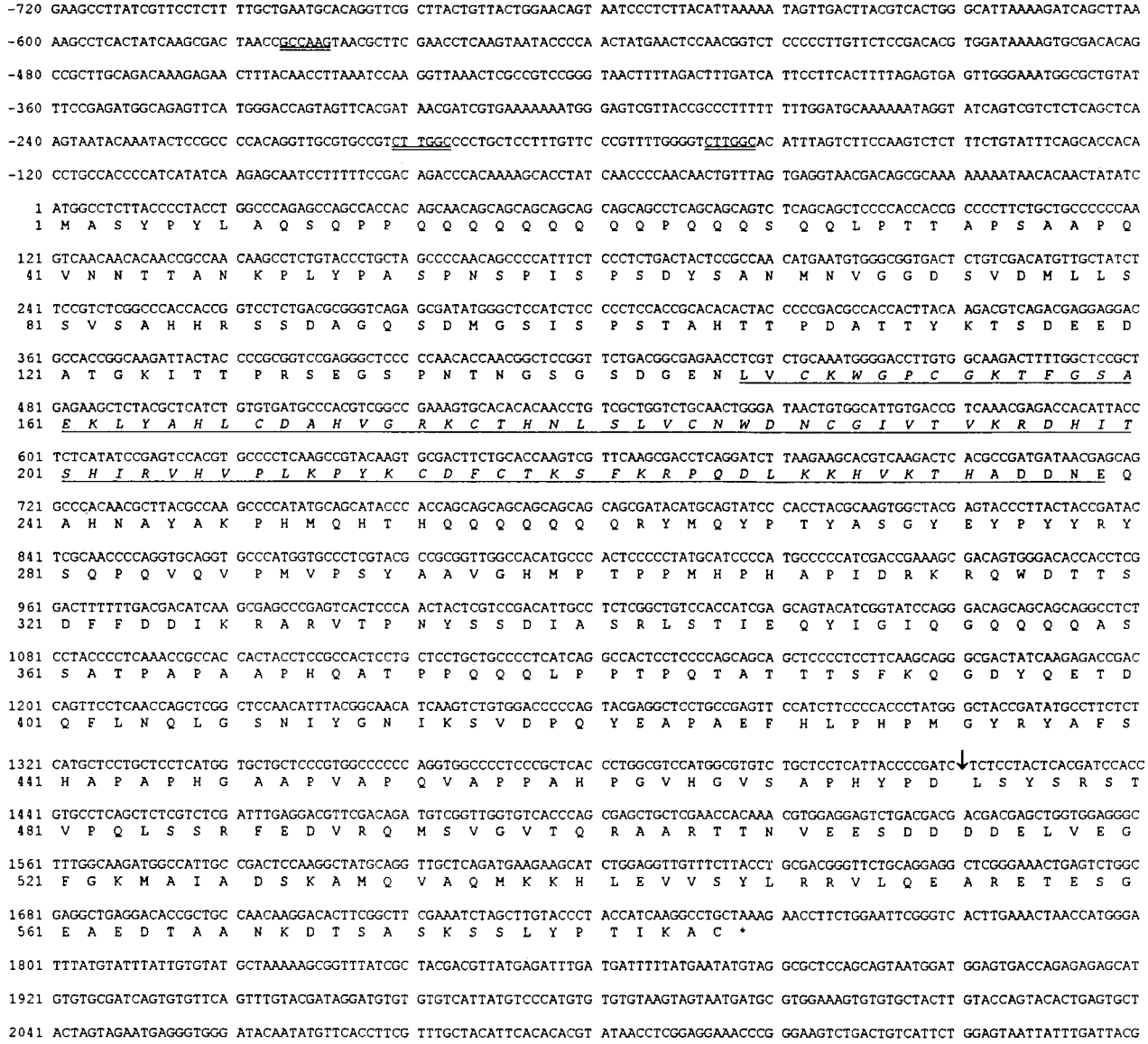

FIG. 4. Nucleotide and derived amino acid sequences of *YlRIM101*. The Zf region is underlined once. Possible YlRim101p binding sites containing the GCCARG consensus (see Table 3) in either orientation in the promoter are underlined twice. The arrow indicates the 3′ truncation point of *YlRIM101-937* and *YlRIM101-1116*.

gested that this truncated form was able to bypass, to a limited extent only, the normal pH regulation of *XPR2*. We reasoned that this might reflect both mRNA instability and the persistence of a significant C-terminal extension beyond the putative proteolytic site.

To support this hypothesis, we tested two additional truncations: the *YlRIM101-5* allele present in strain PH5 (see reference 7 and Materials and Methods), which truncates the protein at position 419 through the introduction of a stop codon in the full-length messenger, and the *YlRIM101-1119* allele (Fig. 2D), which truncates the mRNA (and the protein at position 330) through the introduction of a stop codon and a transcriptional terminator (strain AM319ΔC; see Materials and Methods). Both constructs replace the wild-type *YlRIM101* gene at its normal location. Northern analysis (Fig. 3B) showed that the full-length RNA present in PH5 was more abundant than the truncated version. Both alleles resulted in a significant increase of *XPR2* mRNA levels at alkaline pH and in a strong derepression at acidic pH. Both alleles resulted in complete repression of AXP transcription at acidic pH, suggesting that these truncated forms activate *XPR2* and repress *AXP* transcription.

In these mutants, *XPR2*-driven β-galactosidase activity is strongly derepressed at acidic pH (Table 1). Although the *YlRIM101-1119* gene product appeared less efficient than the wild-type product at alkaline pH (which may reflect too extensive truncation), its effects on its own transcription and on *XPR2* appear completely pH insensitive.

Effects of *PAL* and *YlRIM101* mutations on growth, mating, and sporulation. To study effects on growth, mating, and sporulation, nearly isogenic strains of opposite mating types were constructed by conventional genetic crosses of strains carrying mutated *PAL* or *YlRIM101* alleles.

We first checked the effects of *pal* mutations and of the null

```
          f C   r  s     m h   t   Position
          12123451234123456123123451234 56
          YxCxx--CGKaFxxxsxLxxHxxx-HTGEKP Consensus
          F
Sc-1      LvCkWdnCgmifnqPElLYnHlChdHVGRKShkNLq
Yl-1      LvCkWnpCgktfgsaEkLYaHlCdaHVGRKcThNLs
An-1      LtCmWqGCsEKlPtPEsLYEHVCERHVGRKSTNNLN
Pc-1      LpCqWvGCtEKsPtaEsLYEHVCERHVGRKSTNNLN
Ag-1      LsClWqGCsEKcPsPEaLYEHVCERHVGRKSTNNLN

Sc-2      LnChWGdCtTkTeKRDHITSHlRV-HVPLKP
Yl-2      LvCnWdnCgivTVKRDHITSHIRV-HVPLKP
An-2      LTCQWGsCrTTTVKRDHITSHIRV-HVPLKP
Pc-2      LTCQWGtCnTTTVKRDHITSHIRV-HVPLKP
Ag-2      LTCQWGsCrTTTVKRDHITSHIRV-HVPLKP

Sc-3      fgCst--CsKkFKRPQDLKKHlKi-Hlesgg
Yl-3      yKCDF--CtKsFKRPQDLKKHVKT-HADDne
An-3      HKCDF--CGKAFKRPQDLKKHVKT-HADDSv
Pc-3      HKCDF--CGKAFKRPQDLKKHVKT-HADDSe
Ag-3      HKCDF--CGKAFKRPQDLKKHVKT-HADDSv
```

FIG. 5. Alignment of the Zf regions from Rim101p/PacC homologs. Zfs from *S. cerevisiae* (Sc) and *Y. lipolytica* (Yl) and PacC of *A. nidulans* (An), *P. chrysogenum* (Pc), and *A. niger* (Ag) were aligned with the consensus sequence of Jacobs (23). Putative zinc-chelating cysteine and histidine residues are in boldface, uppercase letters. Lower- and uppercase letters in the consensus indicate specific residues that display ≥50 to 80% and ≥80% conservation, respectively (23). In Rim101p/PacC Zf, uppercase letters indicate residues conserved in at least three of the five Zfs; underlined letters denote identity with the consensus.

allele *Ylrim101-1113* on growth rates on YPD medium at pHs ranging from 3.5 to 8.0. Whatever the pH of the culture medium, growth curves were superimposable on the curve of strain AM3, indicating that neither the *pal* mutations nor the *YlRIM101* null mutation significantly affected the growth rate.

As shown in Table 2, all *pal* mutants mated less efficiently than the control strains, yielding few *pal*/+ and even fewer *pal*/*pal* diploids. To test if mating could be restored by a suppressive form of YlRim101p, we transformed one of the *pal* parents with pINA937 (Fig. 2A). As shown in Table 2, mating efficiency was then comparable to that observed with the corresponding *PAL*$^+$ parent. This suggests that mating in *Y. lipolytica* requires activation of *YlRIM101* in one of the parents at least. To test the effect of *pal* mutations on sporulation, homozygous *pal* diploids were cured of pINA937. Cured diploids formed no or very few asci, whereas sporulation was restored to *pal1* and *pal3* diploids still carrying the suppressor. This indicates that *pal* mutations block sporulation in *Y. lipolytica* and that this defect can be bypassed in some *pal* contexts, at least, by expressing a truncated form of YlRim101p.

To test directly the requirement for YlRim101p in mating and sporulation, we studied crosses involving either strains with *YlRIM101* deleted or strains expressing truncated versions of it in a *PAL*$^+$ context. As shown in Table 2, strains with null mutations in *YlRIM101* or a deletion of the Zf region were completely unable to mate with either *YLRIM101*$^+$ or *Ylrim101* mutant strains. Introduction of a plasmid (pINA1120) expressing the wild-type *YlRIM101* gene into the deleted strains fully restored efficiency of mating with the wild-type strain but did not restore efficiency of mating with a *Ylrim101* mutant partner. This shows that expression of *YlRIM101* is absolutely required in both partners for mating. We then tested whether *YlRIM101* is required for sporulation by chasing out the pINA1120 plasmid from the diploids. Whereas *YlRIM101*$^+$ diploids sporulated normally, cured strains failed completely to sporulate. This shows that *YlRIM101* is required for sporulation. We finally checked if all *YlRIM101* effects could be mediated through the truncated form produced by the strong suppressor *YlRIM101-1119* (Fig. 2D and Table 2). Strains expressing only *YlRIM101-1119* mated efficiently with wild-type controls and with some delay with a *YlRIM101-1119* partner. Sporulation of the diploids was very efficient in all cases, suggesting that the truncated form of *YlRIM101* is able to fulfill all YlRim101p requirements for mating and sporulation.

DISCUSSION

We isolated recessive mutations preventing *XPR2* derepression under conditions of carbon and nitrogen limitation at alkaline pH. They identify four unlinked genetic loci named *PAL1* to *PAL4*. We focused on a dominant suppressor common to all *PAL* mutations encoding a C-terminally truncated version of YlRim101p. Interestingly, a point mutation (previously called *RPH2-5*) truncating YlRIM101 was identified in an independent screen (9) when looking for mutations derepressing alkaline protease at acidic pH. This led to the identification of a new member of the family of Zf transcriptional factors (4, 43), like Rim101p (46), which controls entry into meiosis in *S. cerevisiae*, or PacC factors (26, 47, 49), which are involved in pH response in filamentous fungi. As discussed in more detail below, all of the genes we identified seem to affect both pathways in *Y. lipolytica.*

At first glance, it may seem unexpected that screening for mutations affecting *XPR2* under conditions of carbon and nitrogen limitation would lead to the identification of mutations of the pH transduction pathway. Actually, according to Ogrydziak et al. (35), either carbon or nitrogen starvation alone is sufficient to derepress the *XPR2* promoter; therefore, single mutations affecting any one of these two regulatory pathways might remain derepressed due to the other functional pathway. Under the conditions used (Y medium), it appears that the only way to block derepression of *XPR2* expression was to affect activation by the pH pathway.

Neither *pal* mutations nor the *YlRIM101* null mutation significantly affected growth over the pH range tested (3.5 to 8), suggesting that YlRim101p has no general function involved in cell growth. In contrast, deletion of *pacC* in *A. nidulans* leads to slow growth, poor conidiation, and altered colonial morphology, irrespective of the pH (49), whereas *RIM101* deletions in *S. cerevisiae* result in altered colony morphology, poor sporulation, and cryosensitivity (46). However, we obtained evidence that *PAL* mutations affect mating in haploids and sporulation in diploids. These effects could be suppressed by expressing a truncated form of *YlRIM101*, suggesting that both processes are actually dependent on the activated form of YlRim101p. Accordingly, we observed that strains with *YlRIM101* deleted failed completely to mate and sporulate, whereas a truncated allele of *YlRIM101* restored both phenotypes. The fact that the frequency of mating severely drops in *pal* × *PAL*$^+$ matings and becomes virtually undetectable in *pal* × *pal* crosses suggests that basal levels of YlRim101p present in Pal mutants are near threshold levels for mating. Higher levels seem to be required for sporulation, since all homozygous *pal* diploids failed to sporulate. Lack of suppression of the sporulation defect in some Pal diploids by the truncated product generated by pINA937 probably reflects suboptimal functioning of this particular YlRim101p truncation, which is also unable to turn *XPR2* on completely at acidic

TABLE 2. Effect of *PAL* and *YlRIM101* status on mating and sporulation

Strains	Genotypes[a]	Status[b]	Mating[c]	Sporulation[d]
AM4 × AM3	*B ura3 his1* × *A ura3 lys11*	+/+	+	+
LAM57 × LAM58	*A leu2 lys11* × *B leu2 his1*	+/+	+	+
LAM57 × LAM26-02	*A leu2 lys11* × *B pal1-26 his1*	+/pal1	±	+
LAM57 × LAM27-04	*A leu2 lys11* × *B pal2-27 his1*	+/pal2	±	+
LAM58 × LAM21-03	*B leu2 his1* × *A pal3-21 lys11*	+/pal3	±	+
LAM58 × LAM24-01	*B leu2 his1* × *A pal4-24 ura3*	+/pal4	±	+
LAM26-02 × LAM26-03	*B pal1-26 his1* × *A pal1-26 ura3*	pal1/pal1	−	−*
LAM27-04 × LAM27-01	*B pal2-27 his1* × *A pal2-27 ura3*	pal2/pal2	−?	−*
LAM21-03 × LAM21-02	*A pal3-21 lys11* × *B pal3-21 ura3*	pal3/pal3	−?	−*
LAM24-03 × LAM24-01	*B pal4-24 lys11* × *A pal4-24 ura3*	pal4/pal4	−?	−*
LAM26-03 × LAM64	*A pal1-26 ura3* × *B pal1-26 leu2 his1* + pINA937	pal1/pal1 RIMΔC1	±	+
LAM27-01 × LAM74	*A pal2-27 ura3* × *B pal2-27 leu2 his1* + pINA937	pal2/pal2 RIMΔC1	±	−
LAM21-02 × LAM77	*B pal3-21 ura3* × *A pal3-21 leu2 lys11* + pINA937	pal3/pal3 RIMΔC1	±	+
LAM24-01 × LAM90	*A pal4-24 ura3* × *B pal4-24 leu2 his1* + pINA937	pal4/pal4 RIMΔC1	±	−
AM4 × AM319ΔC	*B ura3 his1* × *A RIM101-1119 ura3 lys11*	+/RIMΔC2	+	+
AM3 × AM419ΔC	*A ura3 lys11* × *B RIM101-1119 ura3 his1*	+/RIMΔC2	+	+
AM319ΔC × AM419ΔC	*A RIM101-1119 ura3 lys1* × *B RIM101-1119 ura3 his1*	RIMΔC2/RIMΔC2	+, 1-day delay	+, 1-day delay
AM3 × AM419ΔC1120	*A ura3 lys11* × *B RIM101-1119 ura3 his1* + pINA1120	+/RIMΔC2 RIM+	+	+
AM4 × AM319ΔC1120	*B ura3 his1* × *A RIM101-1119 ura3 lys11* + pINA1120	+/RIMΔC2 RIM+	+	+
AM319ΔC1120 × AM419ΔC1120	*A RIM101-1119 ura3 lys11* + pINA1120 × *B RIM101-1119 ura3 his1* + pINA1120	RIMΔC2 RIM+/ RIMΔC2 RIM+	+, 1-day delay	+
AM4 × SBR98	*B ura3 his1* × *A rim101-1018 ura3 lys11*	+/rimΔZf	−	−**
AM3 × SBR110	*A ura3 lys11* × *B rim101-1018 ura3 his1*	+/rimΔZf	−	−**
SBR98 × SBR110	*A rim101-1018 ura3 lys11* × *B rim101-1018 ura3 his1*	rimΔZf/rimΔZf	−	−**
AM4 × SBR98 1120	*B ura3 his1* × *A rim101-1018 ura3 lys11* + pINA1120	+/rimΔZf RIM+	+	+
AM3 × SBR110 1120	*A ura3 lys11* × *B rim101-1018 ura3 his1* + pINA1120	+/rimΔZf RIM+	+	+
SBR98 1120 × SBR110 1120	*A rim101-1018 ura3 lys11* + pINA1120 × *B rim101-1018 ura3 his1* + pINA1120	rimΔZf RIM+/ rimΔZf RIM+	+	+
AM4 × FL3ΔR	*B ura3 his1* × *A rim101-1113 ura3 lys11*	+/rimΔ	−	−**
AM3 × SBR109	*A ura3 lys11* × *B rim101-1113 ura3 his1*	+/rimΔ	−	−**
FL3ΔR × SBR109	*A rim101-1113 ura3 lys11* × *B rim101-1113 ura3 his1*	rimΔ/rimΔ	−	−**
AM4 × FL3ΔR 1120	*B ura3 his1* × *A rim101-1113 ura3 lys11* + pINA1120	+/rimΔ RIM+	+	+
AM3 × SBR109 1120	*A ura3 lys11* × *B rim101-1113 ura3 his1* + pINA1120	+/rimΔ RIM+	+	+
FL3ΔR 1120 × SBR109 1120	*A rim101-1113 ura3 lys11* + pINA1120 × *B rim101-1113 ura3 his1* + pINA1120	rimΔ RIM+/rimΔ RIM+	+	+

[a] All strains carry an *XPR2-lacZ* fusion at the *XPR2* locus.
[b] *YlRIM101* alleles are abbreviated as follows: RIM+, wild-type gene carried in the chromosome or on pINA1120; ΔC1, C-terminal truncation carried on replicative plasmid pINA937; ΔC2, C-terminal truncation *YlRIM1-1119* replacing the wild-type *YlRIM101* copy in the genome; ΔZf, Zf deletion *Ylrim101-1018* replacing the wild-type *YlRIM101* copy in the genome; rimΔ, complete deletion *Ylrim101-1113* of the genomic copy.
[c] Mating was scored + when comparable to the control, ± when significantly fewer diploids were obtained, − when no diploids were obtained, and −? when one or two clones were observed in some confrontations only.
[d] Sporulation was scored + when asci appeared as abundant as in a control cross and − when only rare asci were observed. * or ** indicates that diploids were tested after curing of a plasmid-borne *YlRIM101-937* or *YlRIM101*$^+$ allele, respectively.

pH (Table 1). The fact that a more severely truncated form of YlRim101p (*YlRIM101-1119*) was able to trigger efficient mating and sporulation shows that the C terminus of *YlRim101* is not required for either process, in contrast to the *S. cerevisiae* situation (46). This indicates that although they are structurally related, YlRim101p and Rim101p may function quite differently.

Although we have no direct evidence that YlRIM101p is a DNA binding protein, all of our observations are compatible with such a hypothesis. As the Zf regions of the Rim101p/PacC homologs show considerable sequence conservation, they probably recognize very similar DNA sequences. The core consensus site for PacC binding in *A. nidulans* is 5′-GCCARG. This sequence was also shown to specifically bind PacC from *P. chrysogenum* in vitro and occurs in the 5′ upstream regions of three fungal alkaline genes controlled by PacC (Table 3). In *Y. lipolytica*, previous in vivo genomic footprints clearly demonstrated that within the proximal UAS (UAS2) of the *XPR2* promoter, the directly repeated decameric sequence CGCCA AGACG, which includes the core PacC hexanucleotide (underlined), interacts permanently with a protein(s) in vivo (5). Moreover, a recent investigation of the role of UAS2 showed that the decameric repeat is able to confer pH-dependent regulation upon a reporter *LEU2* promoter (27). This strongly suggests that protein binding to the decameric repeat may activate *XPR2* directly in response to alkaline ambient pH. Two copies of this decamer (one with a C instead of a G in 3′) are present at positions −168 and −202 upstream from the *YlRIM101* coding sequence (Table 3). This suggests that *YlRIM101* transcription might be autoregulated in a pH-dependent manner, like its homolog *pacC* in *A. nidulans* (49). Indeed, we observed that *YlRIM101* expression was pH and *PAL* gene dependent (Fig. 3A).

The 5′ upstream region of the *AXP* gene expressed at acidic pH (53) carries no copy of the full decameric sequence but three copies of the core PacC hexanucleotide (Table 3). The significance of these sites is unclear, since a repressing effect of the truncated YlRim101p forms could be evidenced only at

TABLE 3. Alignment of potential Rim101p/PacC binding sites

Organism	Gene(s)	Reference	Sequences
Y. lipolytica	*XPR2*	4	GCCAGGAAT
			GCCAAGACG[a,c]
			GCCAAGACG[a,c]
	RIM1	This work	GCCAAGTAA
			GCCAAGACG
			GCCAAGACC
	AXP	49	GCCAAGCGA
			GCCAAGAAA
			GCCAGGGTC
A. nidulans	*pacC*	45	GCCAAGAAT
			GCCAAGGCA
			GCCAAGCTT
			GCCAGGCGT
	ipnA	45	GCCAGGCGG[b]
			GCCAAGAGG[b,c]
			GCCAAGCGA[b]
			GCCAAGCCC[b]
			GCCAAGAAT[b]
A. niger	*pacC*	23	GCCAAGAGG
			GCCAAGAGG
			GCCAGGATG
P. chrysogenum	*pacC*	43	GCCAAGCGT
			GCCAAGCTT
	pcbAB-pcbC	43	GCCAAGCGG[b]
			GCCAAGTCG[b,c]
			GCCAAGAAC[b,c]
Consensus			GCCARGWSS

[a] Potential YlRim101p binding sites (in vivo dimethyl sulfate footprints).
[b] Confirmed PacC binding sites (in vitro DNase I footprints).
[c] Strong protection. Other sites are putative. Underlined letters correspond to positions matching the derived consensus (R is purine, W is A or T, and S is C or G).

acidic pH, a situation that is not likely to occur under physiological conditions. The somewhat variable effects of *pal* mutations on *AXP* transcription at acidic pH (Fig. 3A) must be reevaluated when null alleles of the *PAL* genes become available.

As mentioned by Tilburn et al. (49), although the hexanucleotide consensus represents an important component of the PacC binding site, it probably is not the whole site. Comparison of the *Y. lipolytica* putative YlRim101p binding sites with the known and putative PacC binding sites (Table 3) leads us to propose a longer consensus for Rim101/PacC strong binding sites: 5′-GCCARGWSS. It should be noted that in *S. cerevisiae*, this consensus and the shorter hexanucleotide core sequence are absent from the 518 bp preceding the *RIM101* coding sequence or the coding sequence of *IME1*, a candidate target of Rim101p (46), suggesting that targets for Rim101p have not been identified in this yeast.

In response to changes in the ambient pH, PacC-type factors are regulated at a posttranslational level in filamentous fungi. Signaling by ambient pH has been extensively studied in *A. nidulans* (7, 13). Sensing of ambient pH is mediated by a series of at least six *pal* genes (2), two of which have been cloned (12, 33). The current model (38) assumes that, at ambient alkaline pH, the 678-amino-acid PacC primary translation product is modified in response to a signal provided by the six *pal* gene products and becomes a substrate for a still unidentified protease which removes about 60% of the polypeptide from its C terminus. This proteolytic event converts PacC into a functional form capable of activating the transcription of genes expressed at alkaline pH and repressing the transcription of genes expressed at acidic pH. The *pacC*^{C} mutations, removing a portion of the carboxyl terminus, appear to allow or mimic proteolysis even in the absence of the *PAL* signal, resulting in continuous synthesis of active PacC irrespective of the ambient pH. In contrast, *pal* mutations prevent the modification, and thus the cleavage, of PacC, resulting in permanently inactive PacC as do *pacC* null mutations. These mutations thus mimic the effects of growth under acidic conditions and result in elevated levels of genes expressed at acidic pH and reduced levels of genes expressed at alkaline pH.

Although experimental data on *Y. lipolytica* are much less complete, several of these features seem to apply to YlRim101p, suggesting that the model may have general significance in yeasts and fungi at least. As in the *Aspergillus* model, YlRim101p is required for turning on transcription of a gene expressed at alkaline pH and its C terminus appears to modulate its transcriptional activity negatively. *PAL* genes appear essential for *XPR2* activation at alkaline pH and seem to act, directly or not, through the C terminus of YlRIM101p, since truncated versions of YlRIM101p appear as gain-of-function, dominant suppressors of *pal* mutations, rendering *XPR2* expression fully independent of all *PAL* gene products at alkaline pH (Table 1). Sequence comparison of PacC and YlRim101p, together with the above results, further suggests that a proteolytic cut may occur around amino acid 330 in YlRim101p at alkaline pH. A mutant with a C-terminal truncation of YlRim101p at this position (allele *YlRIM101-1119*) is completely pH unresponsive, whereas less extensive deletions produce increasing pH susceptibility (Table 1).

Significant differences from the *Aspergillus* model appeared when the regulation of *AXP*, a gene expressed at acid pH, was examined. Whereas the *YlRIM101* null mutation and the four *pal* mutations indeed abolished or strongly reduced *XPR2* expression, they did not lead to full derepression of the *AXP* gene at alkaline pH. Contrary to what is observed in *A. nidulans*, these mutations do not mimic fully acidic growth conditions (Fig. 3).

We can thus conclude that in *Y. lipolytica*, (i) *YlRIM101*, as well as each of the four *PAL* genes, is essential for activation of the *XPR2* gene, which is expressed at alkaline pH; (ii) the truncated form of YlRim101p is able to activate *XPR2* transcription regardless of the pH and of the status of the *PAL* genes; (iii) the truncated form of YlRim101p strongly represses *AXP* transcription at acidic pH, but additional factors seem to be involved in the shutdown of *AXP* transcription at alkaline pH; (iv) YlRim101p is essential for sporulation and mating in a *PAL*-dependent manner, and all of its effects can be mediated by its C-truncated form. Further work is needed to assess whether YlRim101p is a direct effector of *XPR2*, *AXP*, and *YlRIM101* and to assess the function of the *PAL* genes. It is hoped that this will lead to a unifying picture of pH regulation in fungi.

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REFERENCES

1. **Ahearn, D. G., S. P. Meyers, and R. A. Nichols.** 1968. Extracellular proteinases of yeasts and yeast like fungi. Appl. Microbiol. **16:**1370–1374.
2. **Arst, H. N., Jr., E. Bignell, and J. Tilburn.** 1994. Two new genes involved in signalling ambient pH in *Aspergillus nidulans*. Mol. Gen. Genet. **245:**787–790.
3. **Barth, G., and C. Gaillardin.** 1996. *Yarrowia lipolytica*, p. 313–388. *In* K. Wolf (ed.), Nonconventional yeasts in biotechnology. Springer-Verlag KG, Berlin, Germany.
4. **Berg, J. M.** 1990. Zinc finger domains: hypotheses and current knowledge. Annu. Rev. Biophys. Chem. **19:**405–421.
5. **Blanchin-Roland, S., R. R. Cordero Otero, and C. Gaillardin.** 1994. Two upstream activation sequences control the expression of the *XPR2* gene in the yeast *Yarrowia lipolytica*. Mol. Cell. Biol. **14:**327–338.

5a. **Blanchin-Roland, S., et al.** Unpublished data.

6. **Boisramé, A., J. M. Beckerich, and C. Gaillardin.** 1995. Sls1p, an endoplasmic reticulum component, is involved in the protein translocation process in the yeast *Yarrowia lipolytica*. J. Biol. Chem. **271:**11668–11675.
7. **Caddick, M. X., A. G. Brownlee, and H. N. Arst, Jr.** 1986. Regulation of gene expression by pH of the growth medium in *Aspergillus nidulans*. Mol. Gen. Genet. **203:**346–353.
8. **Cohen, B. L.** 1973. Regulation of intracellular and extracellular neutral and alkaline proteases in *Aspergillus nidulans*. J. Gen. Microbiol. **79:**311–320.
9. **Cordero Otero, R., and C. Gaillardin.** 1996. Dominant mutations affecting expression of pH-regulated genes in *Yarrowia lipolytica*. Mol. Gen. Genet. **252:**311–319.
10. **Cutler, J. E.** 1991. Putative virulence factors of *Candida albicans*. Annu. Rev. Microbiol. **45:**187–218.
11. **Davidow, L. S., M. M. O'Donnell, F. S. Kaczmarek, D. A. Pereira, J. R. DeZeeuw, and A. E. Franke.** 1987. Cloning and sequencing of the alkaline extracellular protease gene of *Yarrowia lipolytica*. J. Bacteriol. **169:**4621–4629.
12. **Denison, S. H., M. Orejas, and H. N. Arst, Jr.** 1995. Signaling of ambient pH in *Aspergillus* involves a cysteine protease. J. Biol. Chem. **270:**28519–28522.
13. **Dorn, G.** 1965. Genetic analysis of the phosphatases in *Aspergillus nidulans*. Genet. Res. **6:**13–26.
14. **Fabre, E., J. M. Nicaud, M. C. Lopez, and C. Gaillardin.** 1991. Role of the proregion in the production and secretion of the *Yarrowia lipolytica* alkaline extracellular protease. J. Biol. Chem. **266:**3782–3790.
15. **Fabre, E., C. Tharaud, and C. Gaillardin.** 1992. Intracellular transit of a yeast protease is rescued by trans-complementation with its prodomain. J. Biol. Chem. **267:**15049–15055.
16. **Fairhead, C., and B. Dujon.** 1994. Transcript map of two regions from chromosome XI of *Saccharomyces cerevisiae* for interpretation of systematic sequencing results. Yeast **10:**1403–1413.
17. **Fournier, P., A. Abbas, M. Chasles, B. Kudla, D. M. Ogrydziak, D. Yaver, J. W. Xuan, A. Peito, A. M. Ribet, C. Feynerol, F. He, and C. Gaillardin.** 1993. Colocalization of centromeric and replicative functions on *ARS* isolated from the yeast *Yarrowia lipolytica*. Proc. Natl. Acad. Sci. USA **90:**4912–4916.
18. **Germaine, G. R., and L. M. Telefson.** 1981. Effect of pH and human saliva on protease production by *Candida albicans*. Infect. Immun. **31:**598–607.
19. **Hanahan, D.** 1985. Technics for transformation of *Escherichia coli*, p. 109–135. *In* D. M. Glover (ed.), DNA cloning: a practical approach, vol. I. IRL Press, Oxford, England.
20. **He, F., J. M. Beckerich, and C. Gaillardin.** 1992. A mutant of 7SL RNA in *Yarrowia lipolytica* affecting the synthesis of a secreted protein. J. Biol. Chem. **267:**1932–1937.
21. **Hoffman, C. S., and F. Winston.** 1987. A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene **57:**267–272.
22. **Hube, B., M. Monod, D. A. Schofield, A. J. P. Brown, and A. R. Gow.** 1994. Expression of seven members of the gene family encoding secretory aspartyl proteases in *Candida albicans*. Mol. Microbiol. **14:**87–89.
23. **Jacobs, G. H.** 1992. Determination of the base recognition positions of zinc fingers from sequence analysis. EMBO J. **11:**4507–4517.
24. **Jarai, G., and F. Buxton.** 1994. Nitrogen, carbon, and pH regulation of extracellular acidic proteases of *Aspergillus niger*. Curr. Genet. **26:**238–244.
25. **Lemesle-Varloot, L., B. Henrissat, C. Gaboriaud, V. Bissery, A. Morgat, and J. P. Mornon.** 1990. Hydrophobic cluster analysis: procedures to derive structural and functional information from 2-D-representation of protein sequences. Biochimie **72:**555–574.
26. **MacCabe, A. P., J. P. Van den Hombergh, J. Tilburn, H. N. Arst, and J. Visser.** 1996. Identification, cloning and analysis of the *Aspergillus niger* gene *pacC*, a wide domain regulatory gene responsive to ambient pH. Mol. Gen. Genet. **250:**367–374.
27. **Madzak, C., S. Blanchin-Roland, R. Cordero Otero, and C. Gaillardin.** Unpublished data.
28. **Maftahi, M., J. M. Nicaud, H. Levesque, and C. Gaillardin.** 1995. Sequencing analysis of a 15.4kb fragment of yeast chromosome XIV identifies the *RPD3*, *PAS8* and *KRE1* loci, and five open reading frames. Yeast **11:**567–572.
29. **Maftahi, M., C. Gaillardin, and J. M. Nicaud.** 1996. Sticky-end polymerase gene disruption in *Saccharomyces cerevisiae*. Yeast **12:**859–868.
30. **Matoba, M., J. Fukayama, R. A. Wing, and D. M. Ogrydziak.** 1988. Intracellular precursors and secretion of alkaline extracellular protease of *Yarrowia lipolytica*. Mol. Cell. Biol. **8:**4904–4916.
31. **Matoba, S., and D. M. Ogrydziak.** 1989. A novel location for dipeptidyl aminopeptidase processing sites in the alkaline extracellular protease of *Yarrowia lipolytica*. J. Biol. Chem. **264:**6037–6043.
32. **Monod, M., G. Togni, B. Hube, and D. Sanglard.** 1994. Multiplicity of the genes encoding secreted aspartic proteinases in *Candida* species. Mol. Microbiol. **13:**357–368.
33. **Negrete-Urtasun, S., S. H. Denison, and H. N. Arst, Jr.** 1997. Characterization of the pH signal transduction pathway gene *palA* of *Aspergillus nidulans* and identification of possible homologs. J. Bacteriol. **179:**1832–1835.
34. **Nelson, G., and T. W. Young.** 1986. Yeast extracellular proteolytic enzymes for chill-proofing beer. J. Inst. Brew. **92:**599–600.
35. **Ogrydziak, D. M., A. L. Demain, and S. R. Tannenbaum.** 1977. Regulation of extracellular protease production in *Candida lipolytica*. Biochim. Biophys. Acta **497:**525–538.
36. **Ogrydziak, D. M., and R. K. Mortimer.** 1977. Genetics of extracellular protease production in *Saccharomycopsis lipolytica*. Genetics **87:**621–632.
37. **Ogrydziak, D. M.** 1993. Yeast extracellular proteases. Crit. Rev. Biotechem. **13:**1–55.
38. **Orejas, M., E. A. Espeso, J. Tilburn, S. Sarkar, H. N. Arst, and M. Peñalva.** 1995. Activation of the *Aspergillus* PacC transcription factor in response to alkaline ambient pH requires proteolysis of the carboxy-terminal moiety. Genes Dev. **9:**1622–1632.
39. **Pavletich, N. P., and C. O. Pabo.** 1991. Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 A. Science **252:**809–817.
40. **Pavletich, N. P., and C. O. Pabo.** 1993. Crystal structure of a five-finger GLI-DNA complex: new perspectives on zinc fingers. Science **261:**1701–1707.
41. **Ross, I. K., F. De Bernardis, G. W. Emerson, A. Cassone, and P. A. Sullivan.** 1990. The secreted aspartate proteinase of *Candida albicans*: physiology of secretion and virulence of proteinase-deficient mutant. J. Gen. Microbiol. **136:**687–694.
42. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
43. **Schuh, R., W. Aicher, U. Gaul, S. Cote, A. Preiss, D. Maier, E. Seifert, U. Nauber, C. Schroder, R. Kemler, and H. Jackle.** 1986. A conserved family of nuclear proteins containing structural elements of the finger protein encoded by Kruppel, a Drosophila segmentation gene. Cell **47:**1025–1032.
44. **Sherman, F., G. R. Fink, and J. B. Hicks.** 1986. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
45. **Simms, P. C., and D. M. Ogrydziak.** 1981. Structural gene for the alkaline protease of *Saccharomycopsis lipolytica*. J. Bacteriol. **145:**404–409.
46. **Su, S. Y., and A. P. Mitchell.** 1993. Molecular characterization of the yeast meiotic regulatory gene *RIM1*. Nucleic Acids Res. **21:**3789–3797.
47. **Suarez, T., and M. A. Peñalva.** 1996. Characterization of a *Penicillium chrysogenum* gene encoding a *PacC* transcription factor and its binding sites in the divergent pcbAB-pcbC promoter of the biosynthetic cluster. Mol. Microbiol. **20:**529–540.
48. **Suzuki, M.** 1989. SPXX, a frequent sequence motif in gene regulatory proteins. J. Mol. Biol. **207:**61–84.
49. **Tilburn, J., S. Sarkar, D. A. Widdick, E. A. Espeso, M. Orejas, J. Mungroo, M. Penalva, and H. N. Arst.** 1995. The *Aspergillus PacC* zinc finger transcription factor mediates regulation of both acidic- and alkaline-expressed genes by ambient pH. EMBO J. **14:**779–790.
50. **White, T. C., and N. Agabian.** 1995. *Candida albicans* secreted aspartyl proteinases: isoenzyme pattern is determined by cell type, and levels are determined by environmental factors. J. Bacteriol. **177:**5215–5221.
51. **Yamada, T., and D. M. Ogrydziak.** 1983. Extracellular acid proteases produced by *Saccharomycopsis lipolytica*. J. Bacteriol. **154:**23–31.
52. **Yaver, D. S., S. Matoba, and D. M. Ogrydziak.** 1992. A mutation in the signal recognition particle 7S RNA of the yeast *Yarrowia lipolytica* preferentially affects synthesis of the alkaline extracellular protease: *in vivo* evidence for translational arrest. J. Cell Biol. **116:**605–616.
53. **Young, T. W., A. Wadeson, D. J. Glover, R. V. Quincey, M. J. Butlin, and E. A. Kamei.** 1996. The extracellular acid protease gene of *Yarrowia lipolytica*: sequence and pH-regulated transcription. Microbiology **142:**2913–2921.
54. **Zarkower, D., and J. Hodgkin.** 1992. Molecular analysis of the C. elegans sex-determining gene tra-1: a gene encoding two zinc finger proteins. Cell **70:**237–249.