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Genetic Control of Extracellular Protease Synthesis in the Yeast *Yarrowia lipolytica*

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ABSTRACT

Depending on the pH of the growth medium, the yeast *Yarrowia lipolytica* secretes an acidic protease or an alkaline protease, the synthesis of which is also controlled by carbon, nitrogen, and sulfur availability, as well as by the presence of extracellular proteins. Previous results have indicated that the alkaline protease response to pH was dependent on YlRim101p, YlRim8p/YlPalF, and YlRim21p/YlPalH, three components of a conserved pH signaling pathway initially described in *Aspergillus nidulans*. To identify other partners of this response pathway, as well as pH-independent regulators of proteases, we searched for mutants that affect the expression of either or both acidic and alkaline proteases, using a YlmTn1-transposed genomic library. Four mutations affected only alkaline protease expression and identified the homolog of *Saccharomyces cerevisiae SIN3*. Eighty-nine mutations affected the expression of both proteases and identified 10 genes. Five of them define a conserved Rim pathway, which acts, as in other ascomycetes, by activating alkaline genes and repressing acidic genes at alkaline pH. Our results further suggest that in *Y. lipolytica* this pathway is active at acidic pH and is required for the expression of the acidic *AXP1* gene. The five other genes are homologous to *S. cerevisiae OPT1*, *SSY5*, *VPS28*, *NUP85*, and *MED4*. *YlOPT1* and *YlSSY5* are not involved in pH sensing but define at least a second protease regulatory pathway.

PROTEASES are secreted by many fungi (Ogrydziak 1993) and are critical components of their ability to colonize various habitats, ranging from food products to living animals and plants (Pavlukova *et al.* 1998). Protease synthesis is in most cases tightly regulated and responds to a combination of environmental stimuli, including nutrient availability, ambient pH, and temperature, all of which also affect cell type in dimorphic fungi (Ogrydziak *et al.* 1977; Glover *et al.* 1997). Due to this complexity, very little is known at present about the signaling pathways that control protease expression in any fungus.

We use as a model of protease synthesis the dimorphic yeast *Yarrowia lipolytica*, which secretes two major extracellular proteases under conditions of carbon, nitrogen, and sulfur starvation (Ogrydziak *et al.* 1977). Whereas both proteases are similarly induced at the end of the exponential phase on complex media containing proteins, the type of protease synthesized is strictly dictated by ambient pH. At acidic pH, induction of the *AXP1* gene leads to secretion of an acid protease (Axp), whereas at neutral pH, an alkaline protease (Aep) is produced as the *XPR2* gene becomes induced (Glover *et al.* 1997). The promoters of these genes do not show any obvious elements in common, except for potential YlRim101p DNA-binding sites that were shown to be critical for the pH response of *XPR2* (Lambert *et al.* 1997; Madzak *et al.* 1999).

The control of *XPR2* activation in response to neutral pH was shown to depend on a conserved fungal signaling pathway, initially described in *Aspergillus nidulans* (Arst *et al.* 1994) and later extended to several fungi, including *Y. lipolytica* (Lambert *et al.* 1997; Treton *et al.* 2000), *Saccharomyces cerevisiae* (Li and Mitchell 1997; Futai *et al.* 1999), and *Candida albicans* (Ramon *et al.* 1999; Wilson *et al.* 1999; Davis *et al.* 2000; El Barkani *et al.* 2000). In *A. nidulans*, a conserved cascade of six Pal genes (Arst *et al.* 1994; Denison *et al.* 1995; Maccheroni *et al.* 1997; Negrete-Urtasun *et al.* 1997, 1999; Denison *et al.* 1998) activates the transcriptional activator PacC (Denison 2000), homologous to Rim101p in *Y. lipolytica* (Lambert *et al.* 1997) and *S. cerevisiae* (Li and Mitchell 1997) and to Rim101p/Prr2p in *C. albicans* (Ramon *et al.* 1999; Davis *et al.* 2000). At its N terminus PacCp contains a zinc-finger, DNA-binding domain overlapping a nuclear localization signal (Tilburn *et al.* 1995; Mingot *et al.* 2001). It may exist under two states: a full-length form at acidic pH, which is mainly cytoplasmic, and a C-terminally processed form, which is exclusively nuclear (Mingot *et al.* 2001). The transition

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between the two forms is triggered at alkaline pH by the activated Pal pathway, which facilitates the transition of PacCp from a closed form, where its C terminus binds to a bipartite domain just downstream from the zinc finger, to an open, proteolytically sensitive form (Espeso *et al.* 2000). Although the long and the short form are both able to bind PacCp sites *in vitro* (Orejas *et al.* 1995), the current model suggests that only the truncated form is transcriptionally competent: it activates alkaline pH responsive genes (Espeso and Penalva 1996) and represses acidic genes (Espeso and Arst 2000).

Although the *A. nidulans* model seems to be generally applicable to other ascomycetous fungi, it might still be incomplete as suggested by the following observations. First, one or more Rim101p-independent pathways in *C. albicans* and in *S. cerevisiae* were recently suggested to control some alkaline responses (Davis *et al.* 2000; Lamb *et al.* 2001). Second, we suggested that Rim101p binding sites may integrate signals other than pH, including nutrient availability (Madzak *et al.* 1999), whereas Rim101p activation in *S. cerevisiae* was shown to occur in response to a carbon source (Li and Mitchell 1997; Bogengruber *et al.* 1998). Whether these last observations reflect indirect effects of yeast metabolism on ambient pH is still a matter of speculation. Third, few cases of acidic regulation were investigated until now. As expected, the two acidic genes, *gabA* in *A. nidulans* (Espeso and Arst 2000) and *PHR2* in *C. albicans* (Ramon *et al.* 1999; Davis *et al.* 2000), derepress at alkaline pH in strains deleted for *pacC* or its homolog and remain silent at acidic pH in strains expressing a truncated, constitutively active form of PacCp. However, whereas a truncated form of Rim101p did repress, as expected, the expression of the acidic gene *AXP1* at acid pH in *Y. lipolytica*, loss of Rim101p did not promote *AXP1* expression at alkaline pH (Lambert *et al.* 1997).

To get a better insight into the regulation of pH-sensitive genes and to identify new, pH-independent regulators of protease expression, we undertook a systematic search of mutants affecting the expression of either or both acidic and alkaline proteases in *Y. lipolytica*. We observed that mutants simultaneously affecting transcription of both protease genes at all pH values were quite common. Some of these did not affect the pH response but outlined a putative peptide sensing pathway and were shown to be protease specific. Surprisingly, most of the remaining mutants turned out to inactivate various components of the Pal/Rim pathway, suggesting that in *Y. lipolytica* at least this pathway is critical for the expression of both acidic and alkaline proteases.

MATERIALS AND METHODS

Strains: Bacterial strains used for transformation and amplification of recombinant DNA were *Escherichia coli* DH5α and XL1-blue (Stratagene, La Jolla, CA). *Y. lipolytica* strain AM4 (*MAT* B, *ura3-302*, *leu2-270*, *his-1*, *XPR2*, *LEU2*, *XPR2′*:*lacZ*) has been described (Lambert *et al.* 1997). SY12 (*MAT* A, *ura3-302*, *leu2-270*, *lys11-23*, *XPR2*, *LEU2*, *AXP1′*:*gusC*, *XPR2′*:*lacZ*) derives from E122 (Fournier *et al.* 1993) by integrative transformation with plasmid pINA1126 (see below).

Construction of the *AXP1:gusC* fusion: A 2-kb fragment carrying the promoter region and the first two codons of the *AXP1* gene (Young *et al.* 1996) was amplified with *Pfu*I polymerase from genomic DNA, using oligonucleotides SBAXP5M (5′-TCCTCGAGATAATGACGATAA) and SBAXP3V (5′-AGA ACTGCATCTTTAGAATTG), and inserted at the *Srf*I site, 30 bp upstream from the ATG of the *gusC* gene in plasmid pINA1133 (J. M. Nicaud and M. T. Le Dall, unpublished results). The resulting plasmid was digested by *Xho*I and *Sal*I and the fragment carrying the translational *AXP1:gusC* fusion was inserted at the *Sal*I site of pINA404 (Blanchin-Roland *et al.* 1994) between the *LEU2* gene and the *XPR2*:lacZ fusion to give plasmid pINA1126.

Culture media and phenotypic tests: Complete YPD medium, minimal yeast nitrogen base (YNB)-glucose medium, derepressing medium (Y), and protease-inducing medium (YPDm) have been described previously (Sherman *et al.* 1986; Blanchin-Roland *et al.* 1994; Lambert *et al.* 1997), as has the serum medium used to induce hyphae formation (Richard *et al.* 2001). Solid and liquid media were buffered at pH 4.0 (with 0.15 M sodium citrate buffer) or at pH 7.0 (with 0.15 M sodium phosphate buffer). The pH of liquid media was stable within 0.3 pH unit during growth. Lac^+ phenotypes were screened on Y medium supplemented with 70 μg/ml of 5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside (X-Gal) and buffered at pH 7.0. Gus^+ phenotypes were screened on cells grown for 2 days at 28° on Y medium buffered at pH 4, then permeabilized for 5 min with chloroform, and overlaid with a top agar containing 1 mg of 5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid (X-Gluc), 1% bacto agar (Gibco BRL), and 100 mM phosphate buffer pH 7.0. Blue color was detected after 3–12 hr at 30°.

Alkaline extracellular protease activity (Aep^+ phenotype) was screened on colonies spotted on skim-milk plates (Ogrydziak and Mortimer 1977). For the detection of acidic extracellular protease activity (Axp^+ phenotype), a similar test was carried out on a medium buffered at pH 4.0 and containing 2% bovine serum albumin (BSA) neither autoclaved nor filtrated. Clearing zones appeared after 3 days at 28°.

Growth at various pH values was assayed by spotting dilutions of late exponential-phase cultures in YPD liquid medium onto plates buffered either with 0.1 M citrate-phosphate at pH 3.5 or with 0.1 M MOPS for assays at pH 7.0–8.5.

DNA and RNA techniques: Standard molecular genetics techniques were performed essentially as previously described (Sambrook *et al.* 1989). Northern analyses were performed as previously described (Lambert *et al.* 1997) from RNA prepared from cells grown at 2–3 OD_{600nm}, with the RNeasy mini-kit from QIAGEN (Courtaboeuf, France). Hybridized membranes were autoradiographed and analyzed with a Storm PhosphorImager, using the actin transcript as an internal control. Southern blots were carried out from *Sac*I-digested DNA separated on 0.9% agarose gel and transferred onto Hybond N+ nylon membranes (Amersham Pharmacia Biotech). Probes were labeled with the enhanced chemiluminescent direct nucleic acid labeling and detection system (Amersham Pharmacia Biotech). A Perkin-Elmer (Norwalk, CT) Thermal Cycler 9600 was used for PCR reactions. Sequences were obtained on an ABI 373A DNA sequencer. They were assembled and annotated using the GCG package (Madison, WI).

The 3′ ends of the *YlRIM20* and *YlMED4* genes were amplified by PCR walking (Devic *et al.* 1997) on total DNA digested

with *Dra*I, using either PalA3ma (5′-GTTCGGTCTCCCGAG GTC) or Med45man (5′-GCCTGAGCACTCGAAGCTG) as primers, and the Expand Long Template PCR system (Boehringer Mannheim, Mannheim, Germany). The PCR conditions were as in Richard *et al.* (2001) except for the annealing temperature (59°).

Construction of *Y. lipolytica* mutants: As described previously (Richard *et al.* 2001), we used a library of W29 genomic fragments cloned in a pHSS6 derivative and randomly mutagenized in *E. coli* by mTnYl1 (Neuveglise *et al.* 1998), which carries *YlURA3* as a selective marker. Four pools of the library were digested separately with *Not*I and used to transform SY12 by the lithium acetate method, omitting addition of dimethylsulfoxide, which was suspected to increase the frequency of illegitimate integration events (Xuan *et al.* 1988; Richard *et al.* 2001). Transformed cells were plated onto YNB medium supplemented with 0.2% casamino acids (Difco, Detroit).

Isolation and characterization of disrupted loci: Chromosomal fragments flanking YlmTn1 insertion sites were amplified by reverse PCR as previously described (Richard *et al.* 2001) using primers Mat3up (5′-TCCTGACTATGCGGGC TATC) and Mat3down (5′-CCTCTTCACCACCAAAATGC), instead of mtn6 and juan2, and Rmt1 (5′-GGTGAAGTCGTC AATGATGTC), instead of juan1, with mtn1. The sequence of each PCR product was obtained using mtn3 (5′-GCGGCCT TCTTTCTTTGG) or mtn2 (5′-GACGTTCCAGATTACGCTC CGG) primers, and the sequence of the disrupted locus was assembled as described (Richard *et al.* 2001). Extension of the sequence on both sides of mTnYl1 was done by primer walking on both strands. Computer searches were done at http://www.ncbi.nlm.nih.gov/blast/blast.cgi and http://www3.ncbi.nlm.nih.gov/gorf/gorf.html for blastx analysis and open reading frame finding (ORF Finder), respectively.

Random spore analysis: Compatible auxotrophs were crossed onto solid YMC medium and sporulation of diploids was induced on solid citrate synthetic medium (Barth and Gaillardin 1996). Spores were isolated by extraction into paraffin oil, essentially as described previously (Gaillardin *et al.* 1973). Briefly, one loop of sporulating culture was resuspended in 100 μl of 50 mm citrate buffer pH 5.0 and incubated 30 min with 0.5 mg/ml zymolyase 20T at 37°. Then, after addition of 400 μl of 50 mm citrate buffer, the suspension was vortexed vigorously for 2 min. After addition of 200 μl of paraffin oil, spores were extracted by 3 min vortexing followed by separation of the organic phase by centrifugation. Dilutions of the spore suspension were plated onto complete medium (H.-V. Nguyen, personal communication) and colonies were tested on appropriate media.

Nucleotide sequence accession numbers: *YlPHR1* and *YlYMR-110c* partial sequences were identified from plasmids AW0A-A010E01 (AL411343) and AW0AA016E03 (AL412258), respectively. The DNA sequence data for *Y. lipolytica* genes have been deposited at EMBL (see text for accession numbers).

RESULTS

Mutant isolation: To identify potential regulators of protease expression in *Y. lipolytica*, we resorted to a gene tagging strategy using the recently developed mTnYl1-transposed library (Neuveglise *et al.* 1998). This approach relies on the homologous integration of genomic fragments interrupted by a transposon to disrupt target genes (Ross-Macdonald *et al.* 1997). To avoid selecting *cis*-acting mutations, our screen was based on the simultaneous inactivation of pairs of expression cassettes put under the control of the promoter of each of the protease genes expressed by *Y. lipolytica*: the *XPR2* gene (induced under neutral conditions and encoding the alkaline extracellular protease Aep) and the *AXP1* gene (induced under acidic conditions and encoding the acidic extracellular protease Axp). The parental strain SY12 coexpresses Aep and β-galactosidase (*XPR2:lacZ* fusion) under the control of the *XPR2* promoter and Axp and β-glucuronidase (*AXP1:gusC* fusion) under the control of the *AXP1* promoter (see materials and methods). When grown on medium Y containing no rapidly metabolized carbon and nitrogen source such as glucose or ammonia, this strain scores Lac^+ and Aep^+ at pH 7.0; *i.e.*, it turns blue on Y-Xgal7 medium and forms halos on skim-milk plates (Lambert *et al.* 1997). At pH 4.0, it scores Gus^+ and Axp^+; *i.e.*, it turns blue on Y-Xgluc4 medium and forms a halo on BSA plates.

SY12 was mutated using the YlmTn1-transposed genomic library (see materials and methods). Approximately 190,000 transformants were replica plated after 2 days and screened for white or pale blue colonies on Y-XGal7 and on Y-XGluc4. All candidates were subcloned and checked twice for the expression of the reporter cassettes at the relevant pH. A total of 123 mutants were retained and were divided into three phenotypic classes. A first class of four mutants affected only *XPR2* expression: they scored pale blue on Y-XGal7 and formed smaller halos than SY12 on skim-milk plates. A second class of 89 mutants affected both *XPR2:lacZ* and *AXP1:gusC* expression: 75 of them scored Lac^- and Aep^- when grown at pH 7 and Gus^- and Axp^- when grown at pH 4, 12 scored $Aep^{+/-}$ at pH 7 and Axp^- at pH 4, and 2 were Aep^- and $Axp^{+/-}$. The third class of 30 mutants affected only *AXP1* activity: they scored white or pale blue on Y-XGluc4 and formed no or small halos on bovine serum albumin plates.

Characterization of the mutants: Mutant analysis was concentrated on the 93 mutants belonging to the first two classes described above. In a first step, the sequence of the regions flanking the transposon was determined after reverse PCR (see materials and methods).

The insertion sites of a total of 83 clones, or 89% of the candidates, could thus be analyzed. Four insertions affected two previously identified genes and 50 resulted from at least two independent disruptions of the same locus, defining seven new loci. Probes derived from these loci, as well as from previously identified Yl*RIM* genes, were used to check if any of the 10 remaining mutants resulted from an illegitimate integration event into one of these targets. This was the case for 6 of them.

In a second step, genetic linkage between the phenotype and the *URA3*-labeled transposon was confirmed by mating at least one mutant per multiply identified target against a SY12 isogenic strain. Of the 23 remaining mutants, 9 were similarly tested and 5 mutants were retained. These were checked for homologous in-

TABLE 1

Classes of mutants and targeted genes

Relevant phenotype[a]	Affected gene[b]	Homolog in *S. cerevisiae*	Homolog in *C. albicans*[c]	Homolog in *A. nidulans*
Aep$^{+/-}$ Axp^{+} (4)	*YlSIN3* (2)	*SIN3*		
Aep^{-} Axp^{-} (75)	*YlRIM101* (3)	*RIM101*	*CaRIM101/PRR2*	*pacC*
	YlRIM8 (3)[d]	*RIM8*	*CaRIM8/PRR1*	*palF*
	YlRIM9 (1)	*RIM9*	*CaRIM9*	*palI*
	YlRIM13 (16)	*RIM13*	*CaRIM13*	*palB*
	YlRIM20 (12)	*YOR275c*	*CaRIM20*	*palA*
	YlRIM21 (0)[e]	*YNL294c*		*palH*
	YlOPT1 (6)	*OPT1*	*CaOPT1*	
	YlSSY5 (11)	*SSY5*		
	YlVPS28 (2)	*VPS28*		
	YlMED4 (1)	*MED4*		
Aep$^{+/-}$ Axp^{-} (12)	*YlRIM13* (2)	*RIM13*	*CaRIM13*	*palB*
	YlOPT1 (1)	*OPT1*	*CaOPT1*	
	YlNUP85 (2)	*NUP85*		

[a] Number of mutants is in parentheses.
[b] Number of independent insertions is in parentheses.
[c] We suggest that use of a *Ca* prefix for *C. albicans* gene names will simplify their distinction from their *S. cerevisiae* homologs.
[d] Formerly *PAL3*, see Treton *et al.* (2000).
[e] Not identified here, formerly *PAL2*; see Treton *et al.* (2000).

tegration of the disruption cassette either by using one of the amplified borders as a probe in a Southern blot analysis or by PCR using primers derived from the flanking regions, so as to reveal the wild type and/or the disrupted locus. Two yielded the expected pattern. A total of 11 genes were thus unambiguously identified (Table 1) and are analyzed below.

The complete sequence of the disrupted open reading frames (ORFs) was assembled from overlapping flanking regions and by PCR walking on both strands of genomic DNA.

The *Y. lipolytica* components of the Rim pathway and their homologs: Thirty-seven insertions affected five genes encoding proteins similar to conserved components of the pH sensing cascade described in *A. nidulans*, *C. albicans*, and *S. cerevisiae*. A common nomenclature is proposed in Table 1 in order to simplify comparisons among yeast species. Surprisingly, no insertion affected the previously identified *PAL2/YlRIM21/palH* gene (Treton *et al.* 2000), indicating that the mutagenesis was not saturating.

Three mutants carried insertions within the *YlRIM101* gene encoding the pH responding transcriptional activator required for *XPR2* expression (Lambert *et al.* 1997). Two possibly nonindependent insertions truncate the protein at amino acid 229 within the third zinc finger (*Ylrim101-C10* and *-R7*, same transposon orientation); the third interrupts the protein at position 361 (*Ylrim101-T48*).

One of the three mutations affecting the *PAL3/YlRIM8/palF* gene (Treton *et al.* 2000) resulted from an insertion at amino acid 589 (*Ylrim8-C2*). The two others resulted from illegitimate integration and were not mapped.

Eight insertions interrupted a predicted protein sequence of 773 residues (AJ319901) at position 18 (*Ylrim20-C14*), 36 (*Ylrim20-B6* and *-D47*, inverted orientation of the transposon), 77 (*Ylrim20-Y1*), 214 (*Ylrim20-B3*), 271 (*Ylrim20-B4* and *-B9*, same transposon orientation), and 305 (*Ylrim20-D55*). Four more insertions affecting the same ORF were revealed by Southern analysis. The predicted protein shares 35, 29, 27, and 20% identity with *A. nidulans* PalA (Negrete-Urtasun *et al.* 1997), *C. albicans* Rim20p (Davis *et al.* 2000), *S. cerevisiae* YOR275c product, and Bro1p (Nickas and Yaffe 1996), respectively. Comparison of the *Y. lipolytica* predicted amino acid sequence and of its homologs (Figure 1) evidences conservation of coiled-coil regions (positions 431–481, 559–589, and 621–649), but not of the proline-rich motif found exclusively in the C terminus of *S. cerevisiae* Bro1p. This suggests that the *RIM20/palA* homolog of *S. cerevisiae* might be YOR275c rather than *BRO1*.

Ten insertions interrupted a predicted 795-residue protein (AJ277150). The following identity scores were obtained at the protein level: 28% with *A. nidulans* PalBp (Denison *et al.* 1995), 25% with *S. cerevisiae* Rim13p (Futai *et al.* 1999), 26% with *Homo sapiens* PalBp (Futai *et al.* 2001), and 27% with *A. oryzae* PalBp (Yaver *et al.* 2000). The Cys and His residues of the catalytic domain, as well as several other residues, appear well conserved in all PalB/Rim13p proteins. The third residue (Asn) of the calpain catalytic triad (Arthur *et al.* 1995) is replaced by an Asp residue in *S. cerevisiae* Rim13p and YlRim13p. The 10 mutants analyzed here carry inser-

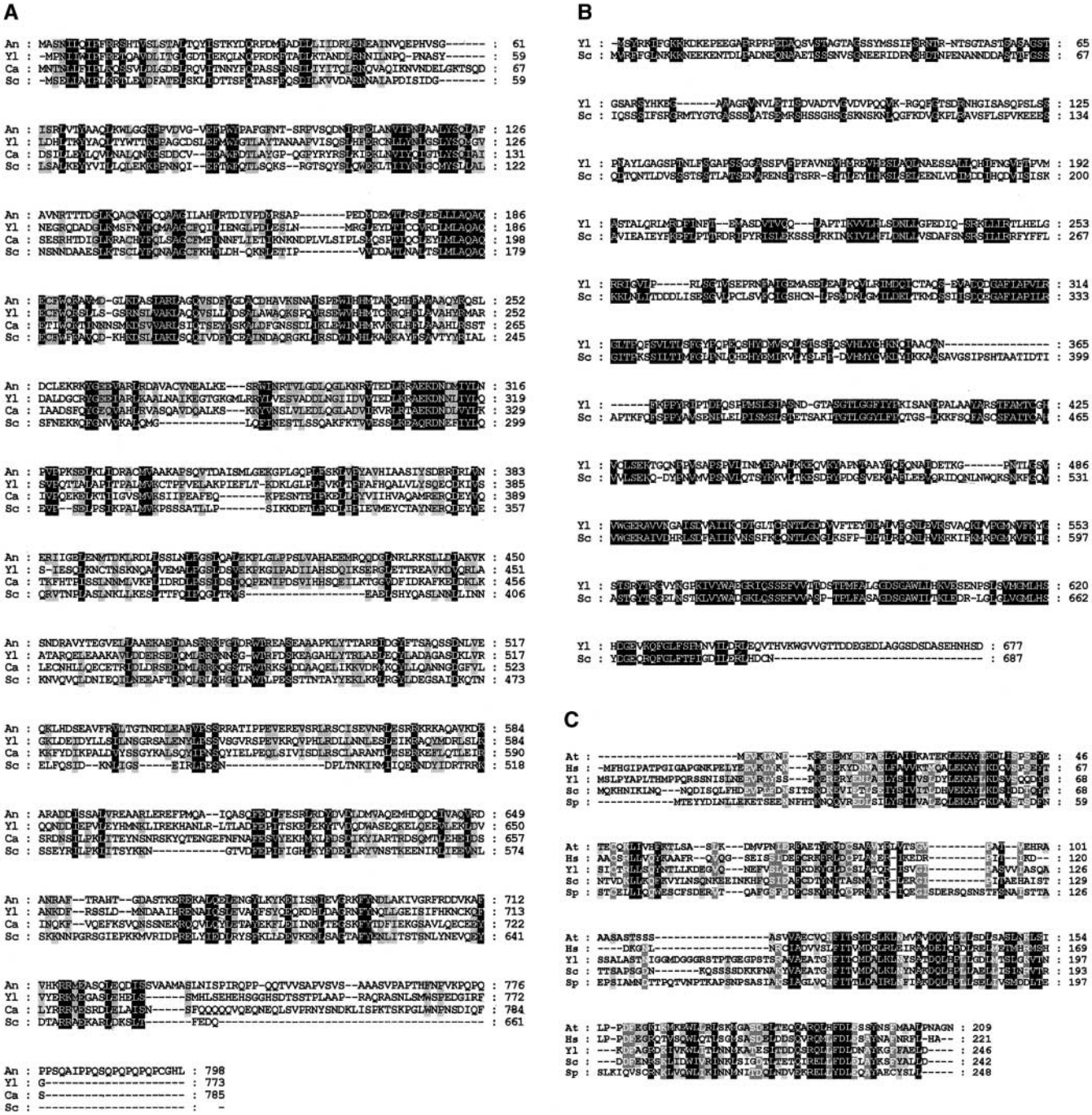

FIGURE 1.—Alignments of *Y. lipolytica* Rim20p (A), Ssy5p (B), and Vps28p (C) with homologous sequences from *S. cerevisiae* (A–C), *A. nidulans* (A), *C. albicans* (A), *A. thaliana, H. sapiens,* and *S. pombe* (C). Sequence alignment was done with the ClustalW algorithm (matrix blosum62) using the nine "strong" groups of conserved residues (THOMPSON *et al.* 1994). (A–C) Amino acid residues that are identical or conserved in all the sequences are shown in white on a black background. (A) Residues identical or conserved in three of these sequences are shown in black on a gray background. (C) Residues identical or conserved in four of these sequences are shown in white on a gray background. Residues identical or conserved in three of these sequences are in black on a gray background.

tions scattered from close to the catalytic site up to the C-terminal region at positions 175 (*Ylrim13-A19*), 202 (*Ylrim13-A21*), 336 (*Ylrim13-A5*), 375 (*Ylrim13-A11*), 493 (*Ylrim13-C12*), 514 (*Ylrim13-A16*), 546 (*Ylrim13-C7*), 663 (*Ylrim13-A10*), 729 (*Ylrim13-A1*), and 743 (*Ylrim13-A41*). Eight more insertions affecting *YlRIM13* were revealed by Southern analysis. All of these 18 mutants exhibited a tight phenotype, except A1 and A41, which scored $Aep^{+/-}$ Axp^{-}, suggesting that the C-terminal end of YlRim13p might be less critical for its activity.

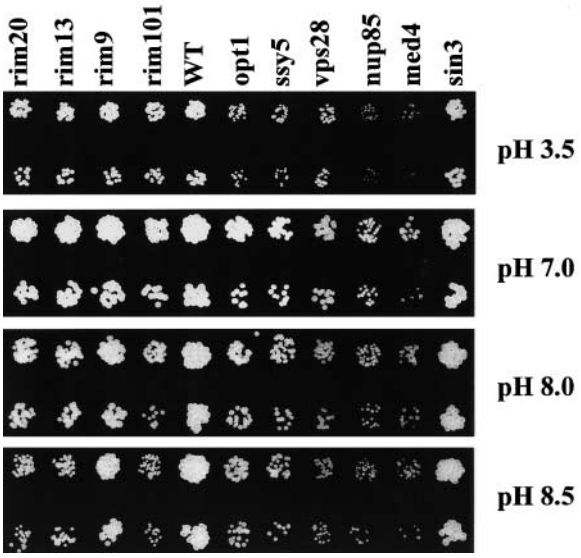

FIGURE 2.—Effects of the different mutations on *Y. lipolytica* sensitivity to ambient pH. Droplets of serial dilutions of a late exponential culture of cells on YPD medium were deposited on solid YPD medium buffered at pH 3.5 with 0.1 M citrate-phosphate buffer and at pH 7.0–8.5 with 0.1 M MOPS buffer and incubated at 28° for 48 hr. The *Y. lipolytica* strains were the following: SY12 (WT), *rim20-C14* (rim20), *rim13-A19* (rim13), *rim9-D7* (rim9), *rim101-C10* (rim101), *opt1-C6* (opt1), *ssy5-B11* (ssy5), *vps28-Y2* (vps28), *nup85-R25* (nup85), *med4-B5* (med4), and *sin3-D18* (sin3).

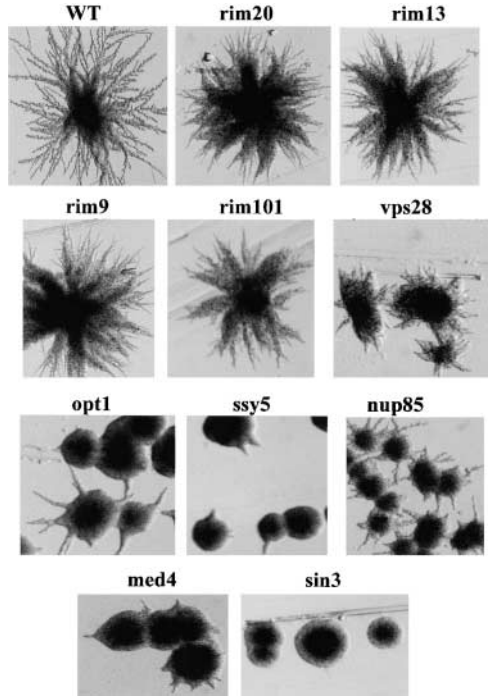

FIGURE 3.—Colonial phenotype of wild-type and mutant strains. Strains were incubated for 2 days on serum medium at 28°. For strain designation, see legend of Figure 2.

One illegitimate integration affected the homolog of *PalI/RIM9* (*Ylrim9-D7*) and was identified by Southern analysis using a probe derived from plasmid AW0AA-029A08 (AL414126), which was reported to carry a partial sequence of *YlRIM9*.

Mutations in *YlRIM101*, *YlRIM9*, *YLRIM13*, and *YLRIM20* affected growth at alkaline, but not at neutral or acidic pH, a phenotype characteristic of Rim mutants. At alkaline pH, *Ylrim101-C10* affected the growth more than *Ylrim20-C14* or *Ylrim13-A19* whereas *Ylrim9-D7* had a less pronounced effect (Figure 2). None of these mutations abolished hyphal formation of the strains (Figure 3), confirming that the Rim pathway is not essential for this transition in *Y. lipolytica* (TRETON *et al.* 2000). We noticed, however, that hyphae were shorter and more branched than in the wild type (Figure 3).

All these mutants simultaneously affected *XPR2* and *AXP1* expression, an unexpected observation that will be discussed below.

Non-Rim components of protease regulation: Seven mutants affected a homolog of *OPT1* of *C. albicans*, which encodes an oligopeptide transporter that exhibits a preference for tetra- and pentapeptides (LUBKOWITZ *et al.* 1997). *YlOPT1* (AJ319902) encodes a predicted protein of 836 amino acids displaying 51% identity with *C. albicans* Opt1p (LUBKOWITZ *et al.* 1997), 41% identity with *Schizosaccharomyces pombe* Isp4p (LUBKOWITZ *et al.* 1998), and 37% identity with *S. cerevisiae* Opt1p (YJL212C; BOURBOULOUX *et al.* 2000). Like other members of the OPT family, YlOpt1p carries 12 potential membrane-spanning segments and a characteristic SPY motif between residues 114–131, before the first hydrophobic domain. One mutant resulted from illegitimate integration and was not mapped. The remaining insertions occurred at positions 269 (*Ylopt1-C6*), 352 (*Ylopt1-B7*), 490 (*Ylopt1-C1*), 730 (*Ylopt1-D14*), 773 (*Ylopt1-Y3*), and 820 (*Ylopt1-Y12*). All these six mutants scored Aep^{-} Axp^{-}; the illegitimate integrant scored $Aep^{+/-}$ Axp^{-}.

A total of 11 mutants, 3 resulting from illegitimate integration, affected a gene (AJ319903) encoding a predicted protein of 677 amino acids displaying 36% identity with *S. cerevisiae* Ssy5p. *S. cerevisiae* Ssy5p is a component of a sensing complex that triggers an intracellular response to the presence of extracellular amino acids (JORGENSEN *et al.* 1998; FORSBERG and LJUNGDAHL 2001). The 22 C-terminal amino acids of YlSsy5p contain nine acidic residues and are thus more acidic than in *S. cerevisiae* Ssy5p (Figure 1). No predicted function could be attributed by the PROSITE program to the conserved regions shared by YlSsy5p and its homolog. Two insertions occurred within the promoter: *Ylssy5-T153* at position −56 from the ATG and *Ylssy5-A58* at position −36. The other insertions interrupt the protein at position 54 (*Ylssy5-B11*), 76 (*Ylssy5-U16*), 165 (*Ylssy5-B12*), 232 (*Ylssy5-B2*), 246 (*Ylssy5-T13*), and 372 (*Ylssy5-B13*). All these mutants scored Aep^{-} Axp^{-}.

Two mutants were affected in a gene homologous to *VPS28* of *S. cerevisiae* and required for protein trafficking to the vacuole through the endocytic and biosynthetic

pathways (Rieder *et al.* 1996). The 1752-bp sequence of the insertion site (AJ319904) evidenced 134 C-terminal codons of an adjacent ORF similar to the *S. pombe* hypothetical gene Spac4G8.04 (36% protein identity). The predicted 246-amino-acid YlVps28 protein matches putative homologous gene products in other species with the following identity scores: 38% with *S. cerevisiae* Vps-28p (Rieder *et al.* 1996), 36% with *Arabidopsis thaliana* F17L22.20 (AL035527) and *S. pombe* Spac1B3.07cp (CAB-11236.1), 35% with *H. sapiens* LOC51160 (AF182844), 34% with *Drosophila melanogaster* CG12770 (AAF59143.1), and 32% with *Caenorhabditis elegans* Y87G2A.10 (CAB-54493.1). Some well-conserved regions between YlVps28p and its homologs were observed (Figure 1), but no clue to their possible function could be obtained using the PROSITE program. Both insertions interrupted the N-terminal part of YlVps28p at positions 87 (*Ylvps28-Y2*) and 98 (*Ylvps28-B1*) and conferred an Aep^{-} Axp^{-} phenotype.

Two mutations affected the homolog of *S. cerevisiae NUP85*, encoding a nonessential nuclear pore protein involved in nuclear-cytoplasmic transport (Goldstein *et al.* 1996). *YlNUP85* encodes a predicted protein of 708 amino acids (AJ315748) displaying 23 and 21% identity, respectively, with *S. cerevisiae* Nup85p (Goldstein *et al.* 1996) and *S. pombe* SpNup85p (AL109846). Both mutants carry the transposon inserted in the central part of YlNup85p at positions 358 (*Ylnup85-R25*) and 473 (*Ylnup85-A54*). They scored $Aep^{+/-}$ Axp^{-}.

A single insertion interrupted a gene (AJ315749) encoding a predicted protein of 336 amino acids, 27% identical to the essential protein *S. cerevisiae* Med4p (Myers *et al.* 1998). The mutant phenotype was genetically linked to the transposon that had inserted by homologous recombination (not shown). *S. cerevisiae* Med4p is a component of the mediator complex of the RNA Pol II holoenzyme (Myers *et al.* 1998). Like *S. cerevisiae* Med4p, YlMed4p displays several acidic stretches in its C-terminal end and a potential coiled-coil domain from amino acid 151 to 196. No possible function could be attributed with the PROSITE program to the regions conserved between YlMed4p and its homolog. The mutation truncates YlMed4p at amino acid position 253 and confers an Aep^{-} Axp^{-} phenotype.

Two insertions affected an ORF (AJ315750) interrupted by a 120-bp intron inserted after the 11th codon. This ORF encodes a predicted protein of 1527 amino acids, displaying 47 and 38% identity with *S. cerevisiae* Sin3p encoding a transcriptional regulator of RNA polymerase II (Wang *et al.* 1990) and with SpPst1p (Dang *et al.* 1999), respectively. Like its homologs, YlSin3p possesses four putative well-conserved PAH (*p*aired *a*mphipathic *h*elix) domains, which may be involved in protein-protein interactions. The *h*istone deacetylase-*i*nteraction *d*omain (HID) located between PAH3 and PAH4 is highly conserved between the three homologs. However, the glutamine-rich segment present within *S. cerevisiae* Sin3p is absent from both YlSin3p and SpPst1p. The *Ylsin3-D18* and *Ylsin3-C13* alleles truncate the protein at positions 342, downstream from the first PAH domain, and 833, within the HID domain, respectively. Both mutants scored $Aep^{+/-}$ Axp^{+}.

Role of the Rim pathway in alkaline induction of gene expression: To confirm the effects of *rim* mutations on the transcription of ambient alkaline pH-responding genes, we searched for a second, nonprotease encoding reporter besides *XPR2*. In *C. albicans*, PHR1 encodes a glycosylphosphatidylinositol-anchored cell surface protein, similar to β-glycosidase (Saporito-Irwin *et al.* 1995). Its expression is restricted to neutral/alkaline ambient conditions and depends upon activation of the PacCp/Rim101p homolog *C. albicans* Rim101p (Ramon *et al.* 1999; Davis *et al.* 2000). A *Y. lipolytica* homolog of *C. albicans PHR1* was identified among the random genomic sequences obtained from W29 (Casaregola *et al.* 2000) and shown to be similarly regulated. To assess the effects of *rim* mutations on *YlPHR1* and *XPR2*, we chose the alleles *Ylrim20-C14*, *Ylrim13-A19*, and *Ylrim101-C10*, which truncate the genes very closely to their 5′ ends, as well as *Ylrim9-D7*, the only mutation affecting *YlRIM9*. Total RNAs extracted from strains grown on Y medium were probed for *XPR2* and *YlPHR1* transcripts. The *XPR2* transcript was detected only in the control strain SY12 grown at pH 7.0 (Figure 4). No expression could be detected at pH 4.0 under these conditions. This confirms that the RIM genes are absolutely required for transcriptional activation of *XPR2* at pH 7.0, but not for transcriptional shutdown at pH 4.0 (Lambert *et al.* 1997). In the *rim* background, the *YlPHR1* mRNA was greatly reduced at pH 7.0 although *Ylrim9* had a milder effect than the other mutants (Figure 4). At pH 4.0, the faint band detected in the control strain SY12 (Figure 4), as well as in the *Ylopt1* and *Ylssy5* mutants (see below), disappeared in the *rim* mutants, suggesting that a residual Rim activity is required to maintain low-level expression of *YlPHR1* at pH 4.0. All these results, also observed from the protease-inducing medium YPDm (data not shown), confirm that the Rim pathway is required for the expression of alkaline-sensitive genes like *XPR2* and *YlPHR1*.

Role of the Rim pathway in acidic induction of gene expression: An unexpected finding was that most mutations impairing both Axp and Aep expression actually affected the Rim pathway. This pathway is known to be essential in *A. nidulans*, *C. albicans*, and *Y. lipolytica* for the induction of alkaline-sensitive genes like *XPR2*, but not for acidic genes like *AXP1*, where only repressive effects of truncated forms of Rim101p/PacCp were documented (Lambert *et al.* 1997; Denison 2000; El Barkani *et al.* 2000).

To check the effect of *Ylrim20-C14*, *Ylrim13-A19*, *Ylrim9-D7*, and *Ylrim101-C10* on the expression of the acidic extracellular protease, Northern analysis was conducted as described above to follow *AXP1* mRNA in cultures grown in Y (Figure 4). In all mutants, as in the wild type, *AXP1* transcription remained undetectable at

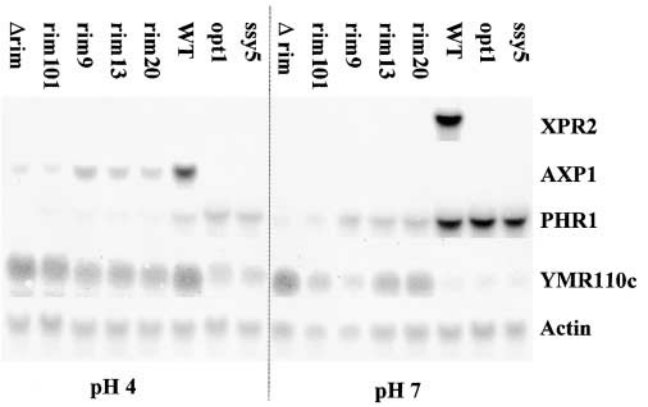

FIGURE 4.—Effects of ambient pH and of the different mutations on *XPR2*, *AXP1*, *PHR1*, and *YMR110c* transcript levels. Strains were grown in Y medium at the indicated pH, and RNAs were extracted. About 37 μg of total RNA was electrophoresed in each case and analyzed by Northern blotting. The probes used are indicated on the right. The alkaline protease gene (*XPR2*) probe was a 0.88-kb fragment from pINA344 (BLANCHIN-ROLAND *et al.* 1994) included in the *XPR2* coding sequence; the acid protease gene (*AXP1*) probe was a 1.39-kb *Eco*RI fragment from JMP50 kindly given by J. M. Nicaud; the *PHR1* probe was a 0.56-kb PCR fragment derived from the coding sequence, kindly provided by B. Treton; the actin probe was a 1.07-kb *Sca*I-*Xho*I fragment from pINA1101 containing the *Y. lipolytica ACT1* gene (S. BLANCHIN-ROLAND, unpublished results); and the *YlYMR110c* probe was a 0.58-kb PCR fragment derived from the coding sequence carried by plasmid AW0AA016E03. RNAs were extracted from wild-type strain SY12 (WT) and from strains carrying the mutations *Ylrim20-C14* (rim20), *Ylrim13-A19* (rim13), *Ylrim9-D7* (rim9), *Ylrim101-C10* (rim101), *Ylrim101-1113* (Δrim), *Ylopt1-C6* (opt1), and *Ylssy5-B11* (ssy5).

pH 7.0, indicating that shutdown of *AXP1* expression at alkaline pH is independent of YlRim101p activation. Surprisingly, however, but in agreement with both β-glucuronidase or acidic protease plate tests, *AXP1* transcription was significantly reduced at acidic pH in all mutants compared to SY12 on Y medium (especially for *Ylrim101-C10*, which destroys the zinc domain of YlRim101p, and for the null allele *Ylrim101-1113*; Figure 4). This shows that the Rim pathway is required for *AXP1* full induction under acidic conditions.

To check if YlRim101p was generally required for acidic and alkaline gene expression, we searched for another acidic gene. Experiments using recently developed *C. albicans* macroarrays (MUNIR *et al.* 2001) evidenced several acidic genes in this species (D. ONESIME, A. LÉPINGLE and C. GAILLARDIN, unpublished results), including a homolog of *YMR110c* recently shown to be also induced by acidic pH in *S. cerevisiae* (CAUSTON *et al.* 2001). A *Y. lipolytica* homolog of this ORF was identified among RSTs of *Y. lipolytica* (CASAREGOLA *et al.* 2000): as shown on Figure 4, this gene is turned on in the wild-type, *ssy5*, and *opt1* strains at pH 4 and turned off at pH 7. However, its expression was largely unaffected by *rim* mutations at acidic pH, whereas it clearly derepressed in these contexts at alkaline pH, especially in a *rim101* deletion background. This shows that, contrary to *AXP1* and as in *A. nidulans*, an acidic gene in *Y. lipolytica* may be Rim independent at acidic pH and Rim repressed at alkaline pH.

A second, pH-independent pathway is involved in expression of the proteases: Contrary to *Rim* mutations, mutations affecting *YlOPT1*, *YlSSY5*, *YlNUP85*, *YlVPS28*, and *YlMED4*, but not *YlSIN3,* did confer a more or less pronounced growth defect either at alkaline and acidic pH (*OPT1*, *SSY5*) or at all pH values tested (*VPS28*, *NUP85*, and *MED4*). None of them showed a specific alkaline-sensitive phenotype (Figure 2). Thus, they do not define new *RIM* genes on this phenotypic basis. Moreover, contrary to *rim* mutations, they do clearly interfere with the ability of the strains to execute the yeast-hyphae transition (Figure 3). On serum-inducing medium, which promotes rapid hyphal formation in the wild type (RICHARD *et al.* 2001), hyphae formation is both strongly reduced and delayed in the mutants. Except in mutants affecting *YlSIN3*, true hyphae are nevertheless formed, suggesting that the morphogenetic pathway itself is still functional but that its response to external stimuli is impaired.

We assessed the effects on the expression of both proteases and *YlPHR1* of mutations interrupting *YlOPT1* (*Ylopt1-C6*) and *YlSSY5* (*Ylssy5-B11*) near the 5′ end of the genes. Both mutations nearly abolished transcription of *XPR2* and *AXP1*, but did not significantly affect the pH-dependent transcription of *YlPHR1* (Figure 4). Thus, *YlOPT1* and *YlSSY5* do not impair pH sensing, but apparently affect another pathway required for protease induction, possibly amino acid sensing.

DISCUSSION

A previous analysis of mutations preventing *XPR2* expression at alkaline pH led to the identification of four genetic loci (*PAL1–PAL4*) involved in ambient pH signaling (LAMBERT *et al.* 1997). Only two genes, *PAL2/YlpalH / YlRIM21* and *PAL3/YlpalF/YlRIM8* (TRETON *et al.* 2000), as well as suppressor forms of *YlRIM101* (LAMBERT *et al.* 1997), could be identified by complementing these *rim* mutations. To improve our understanding of the regulatory pathway and to facilitate identification of the relevant genes, we generated a new library of tagged mutations (NEUVEGLISE *et al.* 1998) affecting expression of Aep at pH 7.0 and/or of Axp at pH 4.0. Approximately 190,000 transformants were screened, corresponding to ~10 insertions/kb of genomic DNA, assuming random mutagenesis and a genome size of 20 Mb for *Y. lipolytica* (CASAREGOLA *et al.* 1997). Of 128 mutants obtained, 94 affected expression of both Aep at pH 7.0 and Axp at pH 4.0. Identification of the disrupted loci by reverse PCR was rather efficient since 86% of the mutants tested could be efficiently processed. Surprisingly, insertions affecting only Aep expression were rare, and all identified the homolog of *S. cerevisiae SIN3*. *S. cerevisiae* Sin3p is a component of the histone

deacetylase B complex, which affects RNA polymerase II transcription and directly or indirectly the expression of a large variety of genes (Wang and Stillman 1993). *YlSIN3* appears to be essential for hyphae formation and important but not essential for *XPR2* expression. Expression of *XPR2* promoter, although low on plates, can be fully induced in liquid YPDm medium (data not shown). Its role on transcription initiation at the *XPR2* promoter remains to be assessed.

Most of the insertions simultaneously impaired Aep and Axp expression. A subset of these represent homologs of the conserved Rim pathway described in *A. nidulans* (Arst *et al.* 1994; Denison *et al.* 1995; Maccheroni *et al.* 1997; Negrete-Urtasun *et al.* 1997, 1999; Denison *et al.* 1998), *C. albicans* (Ramon *et al.* 1999; Wilson *et al.* 1999; Davis *et al.* 2000; El Barkani *et al.* 2000), and *S. cerevisiae* (Li and Mitchell 1997; Futai *et al.* 1999). In addition to the previously identified *YlRIM101*, *YlRIM21*, and *YlRIM8* genes (Lambert *et al.* 1997; Treton *et al.* 2000), three new genes, *YlRIM20*, *YlRIM13,* and *YlRIM9*, were identified in this study. At alkaline ambient pH, all of them are required for normal growth (Figure 2) and for expression of *PHR1* and of *XPR2* (Figure 4). None of them is essential for the yeast-hyphae transition, although they do affect hyphal branching (Figure 3). Interestingly, none of the remaining mutations conferred this set of phenotypes, indicating that no new candidate for a *RIM* gene could be identified. Altogether, six genes are thus identified in the Rim pathway of *Y. lipolytica vs.* seven in *A. nidulans* (Negrete-Urtasun *et al.* 1999). No homolog of the *A. nidulans palC* gene was identified, indicating a library bias or, since *palC* is not conserved in the complete genome of *S. cerevisiae* (Negrete-Urtasun *et al.* 1999), that *palC* homologs either evolved rapidly, were replaced by nonorthologs, or even were lost in yeasts.

Unexpectedly, and contrary to the acidic-mimicking phenotype expected for *rim* mutations, insertions into *YlRIM20*, *YlRIM13*, *YlRIM9*, and *YlRIM101* reduced significantly expression of the acidic gene *AXP1*. A limited effect on *AXP1* expression of point mutations in *YlRIM21* and *YlRIM8* was actually observed in a previous report (Lambert *et al.* 1997), suggesting that the entire Rim pathway is required for optimal *AXP1* expression. The nature of the signal transmitted at acidic pH by the Rim pathway is presently unclear, as well as its target, although Rim101p appears a likely candidate. The strongest effect was indeed observed in the case of *YlRim101-C10*, an insertion in the segment specifying the third zinc finger of YlRim101p, which exhibits a phenotype similar to that conferred by the null allele *rim101-1113* (Figure 4). This suggests that the Rim pathway might still be active at pH 4.0 and that low levels of activated Rim101p, insufficient to turn transcription of *XPR2* on, are required for *AXP1* induction. In keeping with this hypothesis, we notice that the alkaline gene *PHR1* exhibits a residual expression at pH 4.0, which is entirely abolished in *rim* mutants. We also note that effects of *rim* mutations on *AXP1* expression were less pronounced on YPDm (data not shown) than on Y medium at the same pH. A working hypothesis is thus that induction of *AXP1* is not dependent on the Rim pathway for pH sensing but for interpreting other signals, possibly nutrient limitations.

Concerning repression of acidic genes at alkaline pH, several reports show that the artificial expression of a truncated Rim101p/PacCp form is able to repress expression of acidic genes at acidic pH in *C. albicans* (El Barkani *et al.* 2000), *A. nidulans* (Espeso and Arst 2000), and *Y. lipolytica* (Lambert *et al.* 1997). Consequently, acidic genes are usually derepressed at alkaline pH when the Pal/Rim pathway is interrupted: this is the case for the *pacA* and *gabA* genes in *A. nidulans* (Tilburn *et al.* 1995; Espeso and Arst 2000) and for *PHR2* in *C. albicans* (Porta *et al.* 1999; Davis *et al.* 2000). A similar situation was observed in *Y. lipolytica* for the homolog of the acidic gene *YMR110c*, strongly suggesting that the model is valid across distantly related ascomycetes. This is clearly not the situation observed for *AXP1*, which remains repressed in these contexts. At the present stage, we cannot exclude that the lack of *AXP1* expression at alkaline pH in a Rim background actually reflects a lack of induction: if Axp activity is required to generate specific inducing peptides as reported in *Penicillium roqueforti* for the aspartyl protease ASPA (Gente *et al.* 2001), no inducer would be generated at alkaline pH where the enzyme is inactive. Then, interruption of the Rim pathway would not be sufficient to activate *AXP1* transcription. *AXP1* expression may even be totally independent of pH signaling through the Rim pathway, which may nevertheless be required for responding to environmental signals other than pH, as proposed above for its induction at pH 4.0.

Mutations in five non-*RIM* genes (*YlOPT1*, *YlSSY5*, *YlVPS28*, *YlNUP85*, and *YlMED4*) prevented expression of both *AXP1* and *XPR2*. They depressed hyphal formation on serum but also on YPD medium (Szabo 2001), without abolishing it completely, suggesting that they interfered with dimorphism regulation. The simplest interpretation is that they affect sensing or transmission of environmental signals required for morphogenesis and for protease induction. Identification of *OPT1* or *SSY5* homologs, which, in *S. cerevisiae*, are involved in oligopeptide transport (Hauser *et al.* 2001) and extracellular amino acids sensing (Forsberg and Ljungdahl 2001), respectively, strongly suggests that amino acid sensing is important for protease and hyphae induction independently of ambient pH. Indeed, when either gene was mutated, transcription of both proteases dropped to undetectable levels but expression of the alkaline-sensitive gene *PHR1* was unaffected. Identification of other elements of this pathway, like a homolog of the Ssy1p sensor involved in the regulation of amino acid and peptide transporters (Didion *et al.* 1998; Iraqui *et al.* 1999), may shed light on the nature of the still-elusive peptide inducer(s) of proteases. Mutations affecting general factors like Med4p

(chromatin organization), Nup85p (nuclear export of mRNA), and Vps28p (vacuolar targeting) strongly affected cell growth, dimorphism, and expression of both proteases. Although these phenotypes may reflect broad pleiotropic effects, we notice that in *S. cerevisiae* the YOR275c/*rim20* gene product was reported to interact in a two-hybrid screen with Snf7p/Vps32p and Vps4p, both involved in protein sorting at the prevacuolar endosome step, like Vps28p identified in this screen (Raymond *et al.* 1992; Ito *et al.* 2001). Moreover, Rim13p/PalBp was shown to interact with Snf7p/Vps32p (Ito *et al.* 2001). The nature and significance of this putative link between the vacuole, the Rim pathway, and protease induction remains to be elucidated.

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