EUKARYOTIC CELL, June 2005, p. 999–1008 Vol. 4, No. 6
1535-9778/05/$08.00+0 doi:10.1128/EC.4.6.999–1008.2005

The *RIM101*/*pacC* Homologue from the Basidiomycete *Ustilago maydis* Is Functional in Multiple pH-Sensitive Phenomena

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Received 19 October 2004/Accepted 18 April 2005

A homologue of the gene encoding the transcription factor Rim101 (PacC), involved in pH signal transduction in fungi, was identified in the pathogenic basidiomycete *Ustilago maydis*. The gene (*RIM101*) encodes a protein of 827 amino acid residues, which shows highest similarity to PacC proteins from *Fusarium oxysporum* and *Aspergillus niger*. The gene had the capacity to restore protease activity to *rim101* mutants from *Yarrowia lipolytica*, confirming its homologous function, and was expressed at both acid and neutral pH. Null Δ*rim101* mutants were not affected in the in vitro pH-induced dimorphic transition, their growth rate, resistance to hypertonic sorbitol or KCl stress, and pathogenicity. However, similar to *pacC* (*rim101*) mutants in other fungi, they displayed a pleiotropic phenotype with alterations in morphogenesis, impairment in protease secretion, and increased sensitivity to Na^+ and Li^+ ions. Other phenotypic characteristics not previously reported in fungal *pacC* (*rim101*) mutants (morphological changes, increased sensitivity to lytic enzymes, and augmented polysaccharide secretion) were also observed in *U. maydis* mutants. All these modifications were alleviated by transformation with the wild-type gene, confirming that all were the result of mutation in *RIM101*. These data indicate that the Pal/Rim pathway is functional in *U. maydis* (and probably in other basidiomycetes) and plays complex roles in pH-sensing phenomena, as occurs in ascomycetes and deuteromycetes.

Ustilago maydis, the basidiomycete responsible for corn smut disease, displays a complex life cycle that requires the plant host for completion. In its haploid nonpathogenic phase the fungus grows like yeast. Mating of compatible yeasts (sporidia) gives rise to the formation of a pathogenic dikaryotic mycelium that may invade the host. Abundant mycelial growth and karyogamy occur in the plant, which develops galls or tumors full of dark teliospores (see reference 3). Germination of teliospores produces basidiospores that bud to reinitiate the life cycle (for reviews see references 1, 7, and 27). Dimorphic transition of *U. maydis* in vitro was reported to occur in haploid or diploid strains as a response to the external pH (38). At neutral pH the fungus grows as a homogeneous population of budding yeast, whereas at acid pH it develops the mycelial form.

As occurs with different physical and chemical stimuli, pH of the medium affects the physiological behavior of fungi in many ways. Nevertheless, specific responses may occur as a result of a change in external pH. The most studied responses of fungi to changes in pH include secretion of different enzymes such as proteases and phosphatases, permease induction, and alterations in the production of some secondary metabolites, etc. (reviewed in references 15, 32, and 33). Studies on the mechanism of the regulation of protein secretion by the external pH led to the discovery in *Aspergillus nidulans* of a specialized signal transduction pathway responsive to pH (8). This pathway, named Pal/Rim, involves the action of different proteins that convey the pH stimulus to a key zinc finger transcription factor, PacC (Rim101), which activates transcription of genes induced at alkaline pH and represses genes induced at acid pH (8, 15, 26, 44). Further studies proved the wide distribution of similar (although not identical) pathways in other ascomycetes and deuteromycetes (9, 13, 25, 34, 43). These studies have demonstrated that the function of PacC/Rim101 is more complex than simply regulating the transcription of pH-dependent specific enzymes. Additional processes in which PacC/Rim101 plays a regulatory role include meiosis, morphogenesis, and salt stress tolerance (9, 23, 36, 43; reviewed in references 32 and 33).

Interestingly, the Pal/Rim pathway has also been reported to play a role in *Candida albicans* dimorphism. This fungus grows in the mycelial form at neutral pH, and as pH decreases, a yeast-like morphology is developed (reviewed in reference 30). Mutation of *pacC*/*RIM101* blocks mycelium formation at neutral pH (34) and reduces pathogenesis (12). In contrast, it does not seem to affect the pH-dependent dimorphic transition in *Yarrowia lipolytica*, whose *RIM101* mutants still grow in the mycelial form in serum medium (19).

Considering that the in vitro dimorphic transition of *U. maydis* is regulated by pH, we investigated whether the Pal/Rim pathway was involved in the process. Accordingly, we have proceeded to the isolation and deletion of the *RIM101*/*pacC* homologue gene (*RIM101*). The data obtained show that the Pal/Rim pathway is operative in this basidiomycete, where, as in other fungi, it controls a series of cellular functions but is not involved in dimorphism.

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MATERIALS AND METHODS

Strains and growth conditions. The following microbial strains were used in this study: *Ustilago maydis* wild-type strains FB1 (*a1b1*) and FB2 (*a2b2*) (2); *U. maydis* mutants BMA1 and BMA7 (*a1b1*Δ*rim101*::*hyg*) and BMA2 and BMA4 (*a2b2* Δ*rim101*::*hyg*), isolated in this work; *U. maydis* complemented

TABLE 1. Oligonucleotides used for PCR

Primer	5′-3′ sequence	T_m[a] (°C)	Primer sense
JRH-G181	CGCGAAGCTTGCCGCAACGTCCGCCGGCA	65	Antisense
JRH-G182	GGGGGAATTCCAAATAGTAACGACAAACA	65	Sense
JRH-G173	GGGGATATCATTCCTTTGCCCTCGGACGAGT	65	Antisense
JRH-G174	GCGCGACACGTATGAAAAAGCCTGAACTCACC	65	Sense
1892	AA(G/A)(C/A)GIGA(C/T)CA(C/T)AT(C/T/A)ACI(A/T)(C/G)C	50	Sense
1894	(C/G)(G/A)TG(T/C)TT(T/C)TTIA(G/A)(G/A)TC(C/T)TGIGG	50	Antisense
2075	CTTGCGACGAGAAGG	55	Antisense
2078	TCTGGGCATTCTTGG	55	Sense
2833	CCCTCCAACACCTCCTCT	65	Antisense
G-654	CGCGCGGTACCATGAACCACCTCTCGC	63	Sense
G-655	GCGCGGTACCGCAACGTCCGCCGGCA	63	Antisense

[a] T_m, melting temperature.

strains PGM1 and PGM2 (*a2b2* Δ*rim101*::*hyg*/pUpacC23) and AAP1(*a2b2* Δ*rim101*::*hyg*/pUpacC22), also isolated in this work; *Yarrowia lipolytica* FL3ΔR (*A rim101-1113 ura3 lys11*) and E121 (*A lys11*) (24); *Y. lipolytica* strain ETA1 (A *rim101-1113 lys11*/pUpacC44), isolated in this work; and *Escherichia coli* DH5α, which was used as a host for plasmid amplification.

U. maydis and *Y. lipolytica* strains were maintained at −70°C in 50% (vol/vol) glycerol. *U. maydis* cells were transferred to liquid complex medium (CM) (21) and shaken at 28°C for 2 to 3 days. *Y. lipolytica* was propagated in yeast extract-peptone-glucose medium (5). These cultures were used as inocula for all subsequent experiments. Yeast or mycelial growth of *U. maydis* in liquid minimal medium (MM) (21) was obtained as earlier reported (38). Liquid media were supplemented when necessary with hygromycin (100 to 350 μg/ml) and/or 6 μM carboxin. For *U. maydis* transformant selection, protoplasts were plated on CM (see above) containing 1 M sorbitol plus the appropriate selective agent(s). Skim milk agar plates were used for alkaline protease induction (31). Plate mating assays were carried out as described in reference 2.

Growth rate of fungal strains was measured in liquid medium. Cells (10^6 per ml) were inoculated into MM or CM (150 ml in 500-ml Erlenmeyer flasks), and shaken (180 rpm) in a water bath at 28°C. At intervals, aliquots were withdrawn and cell numbers were measured with a Neubauer cell counting chamber. The effect of different stress conditions on cell growth was measured in plates of solid MM of pH 3 or 7 (46).

PCR conditions and plasmid constructions. Amplifications described were performed using *U. maydis* DNA, and the primers and annealing temperatures are described in Table 1. After an initial denaturing temperature of 94°C for 4 min, 30 cycles of the following program were applied: 1 min at 94°C, 1 min at the annealing temperature (Table 1), and extension time (calculated as 1 min per kb of the expected amplification fragment) at 72°C. This program was followed by 5 min at 72°C for final extension.

A 120-bp fragment containing part of the zinc finger coding region of the *RIM101* gene was amplified by PCR, using the degenerate oligonucleotides 1892 and 1894 (Table 1). This fragment was cloned in the pCR2.1 plasmid (Invitrogen) to generate pUznf16.

A partial fragment of the *RIM101* gene of approximately 3,000 bp was obtained by amplification with oligonucleotides JRH-G182 and JRH-G181 (Table 1) and cloned in pUC19 using EcoRI and HindIII sites included in the primers to generate pUpacC.

Plasmid pUpacH was constructed as follows: plasmid pUpacC was digested with StuI and BamHI, eliminating a 1,289-bp fragment that contained the region encoding the three *RIM101* zinc fingers. The BamHI site was filled with the Klenow fragment (Invitrogen) and an EcoRV-EcoRV fragment from pHyg1 (see below) containing the *E. coli Hph* gene open reading frame (ORF), which confers hygromycin resistance, was cloned in frame at this site. This construction was confirmed by sequencing.

Plasmid pHyg1 was derived from pCR2.1 (Invitrogen) and contained the ORF of the *Hph* gene from *E. coli* obtained by PCR with primers JRH-G174 and JRH-G173 (Table 1).

Plasmid pUpacC4 was derived from pCR2.1 (Invitrogen) and contained a 3,832-bp fragment with the complete *RIM101* ORF and 1,175 bp of the promoter region, as well as 172 bp of the terminator, obtained by PCR with oligonucleotides JRH-G182 and 2833.

Plasmids pUpacC22 and pUpacC23 were derived from pCBX122 carrying the carboxin resistance gene (22). pUpacC22 contained a 3,000-bp EcoRI fragment from pUpacC (promoter and partial ORF of *RIM101*), whereas pUpacC23 contained a 3,832-bp fragment from pUpacC4 (1,175 bp of promoter region, *RIM101* ORF, and 172 bp of terminator).

The plasmid used to transform *Y. lipolytica* with the *U. maydis* gene encoding Rim101 (pUpacC44) was constructed through a series of intermediates. *Y. lipolytica* plasmid pINA404 (5) was digested with BamHI to excise the *lacZ* ORF regulated by the *Y. lipolytica Xpr2* gene promoter and terminator. Next, an 1,809-bp fragment containing a truncated *RIM101* ORF, without the promoter region (amplified with oligonucleotides G-654 and G-655 [both containing a KpnI site; see Table 1]), was cloned at the KpnI site to generate plasmid pRpacC. The following step involved the ligation of an EcoRI-SalI fragment (ca. 3,000 bp) from pRpacC containing the *RIM101* fragment, plus the *Xpr2* gene promoter and terminator, into the same sites of pUC18, to generate plasmid pYpacC. Finally pYpacC was digested with EcoRI and HindIII, and the resulting fragment containing the truncated *RIM101* gene ORF plus the *Xpr2* gene promoter and terminator was cloned in plasmid pINA444 (18), which carries the *Y. lipolytica URA3* gene and a *Y. lipolytica* autonomously replicating sequence.

DNA procedures. DNA was obtained as described in reference 20. Transformation of *U. maydis* protoplasts was performed by standard methods (see reference 45). Transformed cells were recovered and further grown on CM-sorbitol plates (see above) containing 350-μg/ml hygromycin B or 6 μM carboxin as described previously (4, 22, 45). *Y. lipolytica* was transformed by the lithium acetate/LiCl method (5), and transformants were recovered in yeast nitrogen broth medium without amino acids (Difco) supplemented with 1 mM lysine, but not uracil.

Standard procedures were followed for molecular cloning. Southern analyses were made with DNA digested with HincII, using as a probe a 1,289-bp StuI-BamHI *RIM101* fragment. Probes were labeled with [α-^{32}P]dCTP by random priming with the Rediprime II random prime labeling system (Amersham Biosciences). DNA was sequenced with a Big Dye Terminator v3.1 cycle sequencing kit according to the manufacturer's recommendations (Applied Biosystems). Universal and sequence-specific primers (Sigma Genosys) were used. Analyses of sequences were performed using DNASTAR software (DNASTAR, Madison, WI) and the BLAST program at the National Center for Biotechnology Information. Specific analyses of the *RIM101* promoter sequence were done in the BLAST program found at http://www.motif.genome.ad.jp. Sequences in the *U. maydis* genome were searched at http://mips.gsf.de/genre/proj/ustilago.

RNA procedures. RNA was prepared by the Trizol technique (Invitrogen) using the manufacturer's suggested conditions. Northern blot analyses were performed with 25 μg total RNA, using ethidium bromide-stained rRNAs as a loading control. Hybridizations were made at 68°C in Church's buffer [5 mM EDTA, 0.25 M $Na_2HPO_4(7H_2O)$, 1% casein hydrolysate, 7% sodium dodecyl sulfate, 0.17% H_3PO_4] for 12 h, and the membrane was washed twice for 10 min at 68°C with 0.05% sodium dodecyl sulfate (39). As a probe we used a 1,289-bp StuI-BamHI *RIM101* fragment labeled as described above.

Pathogenicity assays in maize plants and teliospore isolation and germination. Assays of pathogenicity of *U. maydis* strains in maize cv. Cacahuazintle plants were made essentially as previously described (28). Plants were inoculated with 0.1-ml aliquots of suspensions containing equal numbers of sexually compatible yeast cells (10^5, 10^4, 10^3, or 10^2 cells/aliquot). Plants were incubated in a greenhouse, and disease symptoms (development of chlorosis, anthocyanin production, and tumor formation) were recorded normally after 15 days. Tumors were excised and treated as described previously (10, 28). After this, teliospores were diluted to 1,000/ml, and 0.1-ml aliquots were distributed on plates of solid

CM. After 12 to 18 h of incubation at 28°C, teliospores had germinated to produce sporidia.

Miscellaneous procedures. Proteolysis in solid medium was measured by the development of clear halos on skim milk agar plates.

The polysaccharide secreted to the culture medium was measured at different times of growth in cultures inoculated with 1×10^6 cells/ml. Cells were eliminated by centrifugation, and ice-cold ethanol (50% final concentration) was added to the cell-free medium. After 12 h at 0°C, the mixture was centrifuged at 3,000 rpm for 5 min, and the precipitated material was dried at ambient temperature and weighed. Its content of neutral sugars was measured by the anthrone procedure (16), using glucose as a standard.

Cell wall sensitivity to *Trichoderma* lysing enzymes (Sigma catalog no. L1412) was determined by protoplast formation. Cells grown in CM were recovered at the end of the log phase by low-speed centrifugation, washed with distilled water twice by centrifugation, and finally resuspended in SCS (1 M sorbitol, 20 mM sodium citrate, pH 5.8). To 1-ml aliquots of SCS containing 10^8 cells, 50 mg of enzyme was added, and every 20 min 10 μl of the mixture was mounted on a glass slide and observed under the microscope. Photographs of different fields were taken, and the ratio of protoplasts to whole cells was determined.

Cells were observed with a Leica DMRE model microscope using phase contrast and photographed with a Spot camera (Diagnostic Instruments). In some experiments cells were stained with a solution containing 0.05 mg/ml of Calcofluor white, thoroughly washed by centrifugation, and observed by epifluorescence. Cell measurements were made with the Image-ProPlus 4.0 program of Media Cybernetics. We measured 30 cells randomly selected from fungal cultures. Frequency of septation was measured per 30 μm of cell length. Statistical significance of data was analyzed by χ^2 determination. Experiments where numerical data were obtained were repeated at least three times with two replicates per experiment.

Nucleotide sequence accession number. The gene sequence has been registered at the EMBL Nucleotide Sequence Database with the accession no. AJ748125.

RESULTS

Isolation of *RIM101* gene. To analyze whether *U. maydis* possessed a homologue of the *RIM101*/*pacC* gene, we used two degenerate primers (1892 and 1894, Table 1), designed on the basis of the amino acid sequences of the most conserved regions (second and third zinc fingers of the coding region) from several *pacC*/*RIM101* homologues, to amplify a putative fragment by PCR. A single product of 172 bp was obtained, whose DNA sequence revealed an ORF with the same consensus of the zinc finger regions as that of other reported PacC/Rim101 proteins (not shown). Using the information on the *U. maydis* genome sequence available from the Center for Genome Research (Cambridge, MA), we designed two specific oligonucleotides (JRH-G182 and 2883 [Table 1]) to amplify the complete ORF and promoter region of the gene homologue of *pacC*/*RIM101*. The amplified fragment of 3,832 bp was cloned in pUC19 and sequenced (pUpacC4). It was found to contain a 5′ region of 1,175 bp, plus a single 2,484-bp ORF with no consensus introns, encoding a protein of 827 amino acid residues with a molecular mass of 88.5 kDa, and including a 3′ fragment of 172 bp. This sequence has been registered at the EMBL Nucleotide Sequence Database with the accession no. AJ748125. It is identical to ORF um10426 from the *U. maydis* genomic bank (http://mips.gsf.de/genre/proj/ustilago). In comparison with the GenBank-deposited sequences from several organisms the best BLAST similarity scores were obtained with PacC proteins of *Fusarium oxysporum* and *Aspergillus niger*. Homology was very high at the zinc finger zone and low at the C-terminal domain (ca. 51 and 22% and 51 and 21%, respectively). In the 5′ region we identified two adjacent putative *A. nidulans* PacC consensus binding sites at positions −614 to −598 from the ATG start codon.

Deletion of the *RIM101* gene and isolation of null mutants. Deletion of the *RIM101* gene was obtained using plasmid pUpacH. The plasmid was linearized with HindIII and used to transform *U. maydis* FB2 and FB1 wild-type strains, to obtain *RIM101* replacement by the *Hph* gene through double-crossover homologous recombination. Under these conditions the *Hph* ORF stays under the control of the *RIM101* gene promoter. This strategy made it possible to analyze later on the transcriptional regulation of *RIM101* by means of hygromycin resistance (see below). Hygromycin-resistant clones were isolated, and homologous recombination was confirmed by PCR with primers 2075 and 2078 (Table 1). These should amplify a 1,662-bp fragment from null mutants (Fig. 1A, lanes 1 to 3) and a 1,925-bp product from wild-type strains (Fig. 1A, lane 4). Since two EcoRI sites were present in the PCR products (Fig. 1D and 1E), these were digested with EcoRI, giving rise to the expected different products in the wild-type and mutant strains (Fig. 1B, lanes 2 and 4 for mutant and lane 6 for wild-type strains). Gene disruption was also confirmed by Southern analysis (Fig. 1C). For this we used the 1,289-bp StuI-BamHI fragment of the *RIM101* ORF as a probe, present only in wild-type strains (Fig. 1D). Two of the transformants obtained from each FB1 and FB2 strain (see Materials and Methods) where homologous gene replacement had occurred were selected for further studies. Strain BMA2 (*a2b2* Δ*rim101*::*hyg*) was used in most of the further analyses.

Analysis of *RIM101* gene expression. As indicated above, expression of the *E. coli Hph* gene, and therefore hygromycin resistance in our Δ*rim101* mutants, was under the control of the *RIM101* promoter (see Materials and Methods). This construction allowed the determination of *RIM101* expression through the hygromycin resistance of the mutants. We observed that these strains, in contrast to wild type, were resistant to hygromycin at concentrations over 150 μg per ml under acid or neutral conditions in different media, providing evidence that *RIM101* was expressed at both acid and neutral pH (not shown). Quantitative determination of expression of the *RIM101* gene was done by Northern blotting. The results obtained (Fig. 2) showed that indeed the transcript was observed at both pH values of growth, although expression was much higher at neutral pH.

Phenotypic analysis of Δ*rim101* mutants. (i) Growth characteristics. The growth rates of BMA2 and BMA4 (mutants) and FB2 (wild-type) strains in liquid CM or MM at pH 7.0 did not display any significant difference (data not shown). Further phenotypic characterization included the use of several stress conditions on solid MM at different cellular concentrations. These included salt stress by addition of 0.6 M KCl, 1 M NaCl, or 0.05 M LiCl at pH 7.0 or 3.0; hyperosmotic stress with 1 M sorbitol; growth at temperature above optimum (32 to 33°C); and incubation in media with different concentrations of Calcofluor white (300 to 500 μg/ml). No significant differences in growth and colonial morphology between the different Δ*rim101* mutants and the wild-type strain were observed under most conditions (not shown). However, exposure to salt stress elicited differential reactions for the mutants and the wild type. Addition of 0.05 M LiCl or 1 M NaCl strongly inhibited the growth of the mutants, in contrast to wild-type strains (Fig. 3). Salt sensitivity occurred only at pH 7 (Fig. 3A and C). KCl had no inhibitory effects at either pH (not shown).

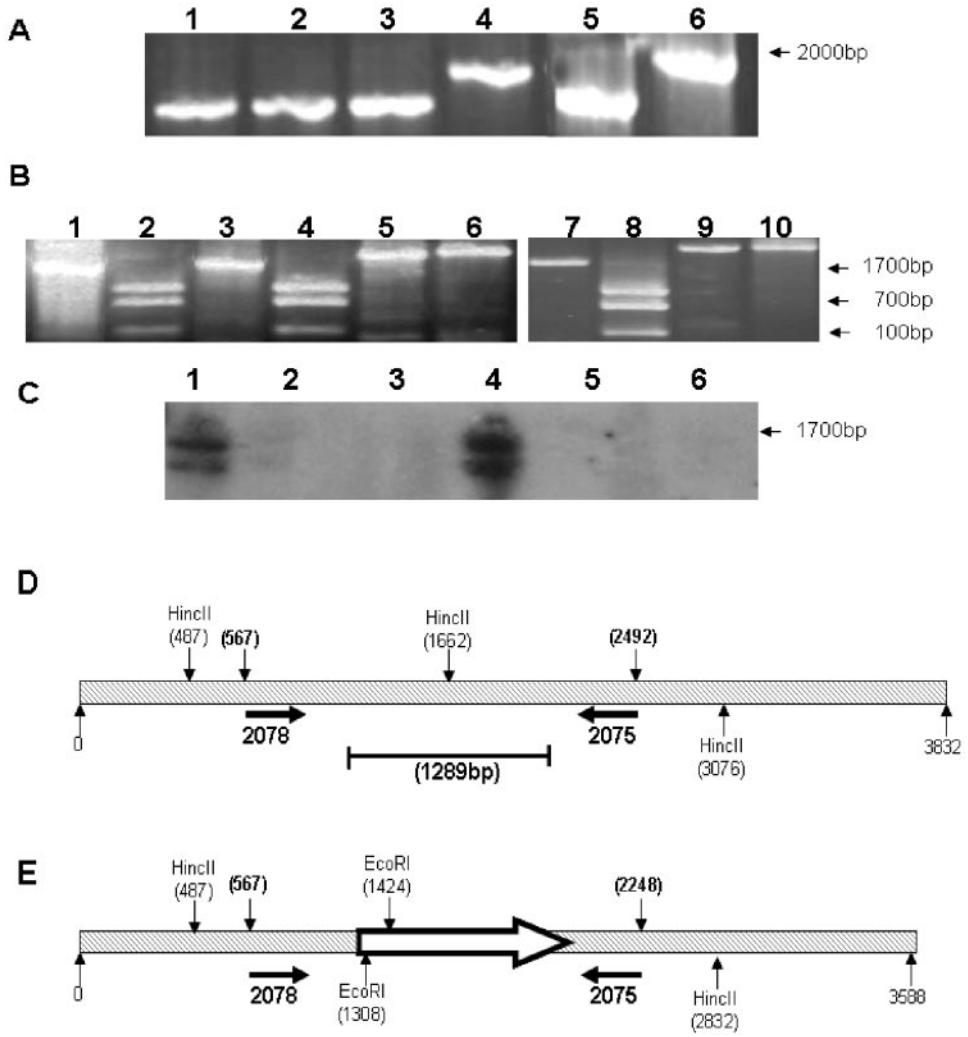

FIG. 1. Confirmation analyses of *RIM101* mutation. A. DNA was subjected to PCR with primers 2075 and 2078. Lanes 1 to 3, Δ*rim101* mutants (BMA1, BMA2, and BMA4, respectively); lane 4, FB2 wild-type strain; lane 5, plasmid pUpacH; lane 6, plasmid pUpacC4. B. EcoRI digestion of PCR products. Lane 2, BMA1; lane 4, BMA2; lane 6, FB2; lane 8, pUpacH; lane 10, pUpacC4. Lanes 1, 3, 5, 7, and 9, controls with no restriction enzyme added to BMA1, BMA2, FB2, pUpacH, and pUpacC4, respectively. C. Southern blot hybridization of HincII-digested DNA with the 1.2-kb StuI-BamHI *RIM101*-specific probe. Lane 1, FB1; lane 2, BMA1; lane 3, BMA7; lane 4, FB2; lane 5, BMA2; lane 6, BMA4. Note absence of hybridization in mutants compared to wild-type strains. Arrows show positions of the molecular size standards. D. Restriction map of *RIM101* locus of the wild-type strain showing the primer (solid arrow) and probe (solid line) positions. E. Restriction map of *RIM101* locus of the mutant allele used in the experiment showing the position of the primers (solid arrows) and the *Hph* gene (large open arrow).

(ii) Dimorphic transition and cellular morphology. We observed that the several Δ*rim101* mutants analyzed were not affected in their dimorphic capacity (Fig. 4). Nevertheless, mycelial morphology and the distribution of septa were different from those of the wild-type strain (Fig. 4A and B). Mycelia of Δ*rim101* mutant BMA2 displayed a significantly higher relative number of septa than did the wild-type FB2 strain (Table 2). Also mutant yeast cells were significantly longer (Table 2), and some of them presented septa (Fig. 4D and E). In contrast, the width of yeast cells was indistinguishable in the mutant (2.83 ± 0.38 μm) and wild-type (2.96 ± 0.38 μm) strains (χ^2 value of 1).

(iii) Cell wall sensitivity to lytic enzymes and polysaccharide secretion. When performing transformation experiments with the standard protocol, we observed that protoplast formation

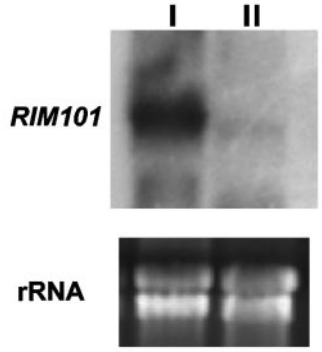

FIG. 2. Determination of *RIM101* expression. Shown are results of Northern analysis of *RIM101* gene expression in strain FB2 grown for 24 h in MM, pH 7 (lane I) or pH 3 (lane II), using the 1.2-kb StuI-BamHI *RIM101*-specific probe. rRNAs stained with ethidium bromide are shown as a loading control.

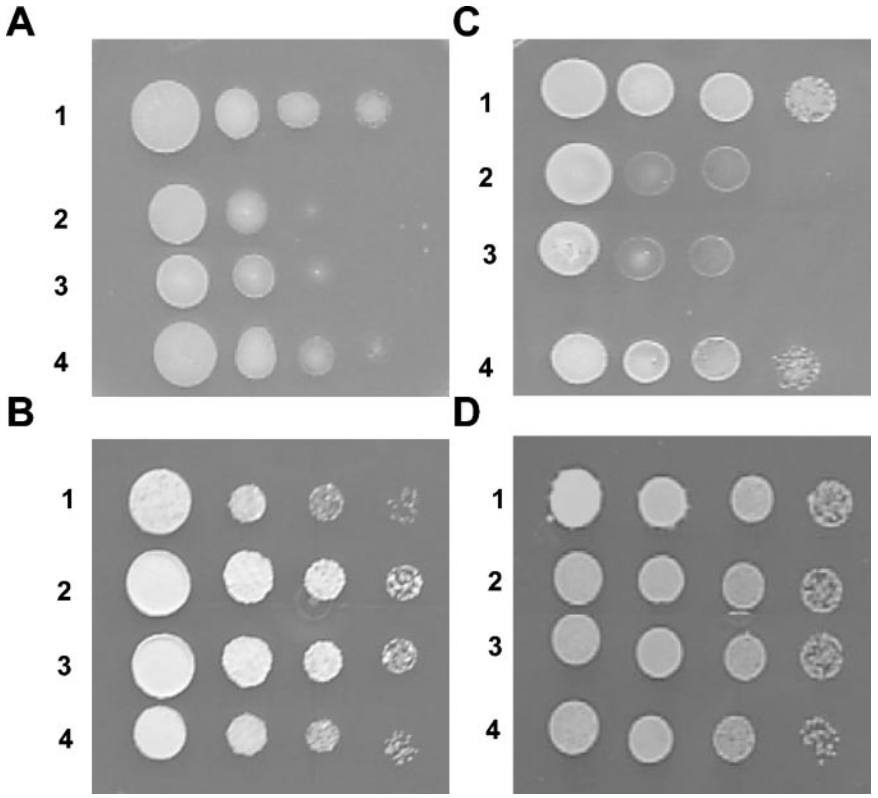

FIG. 3. Effect of salt stress on *U. maydis* growth. Different *U. maydis* cell numbers (10^7, 10^6, 10^5, or 10^4 cells/aliquot) were inoculated on plates of solid MM and incubated for 96 h. Panels A and C, MM, pH 7; panels B and D, MM, pH 3. Panels A and B, 0.05 M LiCl added to the medium; panels C and D, 1 M NaCl added to the medium. Rows 1, FB2; rows 2, BMA1 (Δ*rim101*); rows 3, BMA2 (Δ*rim101*); rows 4, PGM1 (Δ*rim101*::*hyg*/pUpacC23).

occurred faster in the Δ*rim101* mutants than in the wild-type strains. Accordingly, we proceeded to perform quantitative experiments and verified that cell wall sensitivity to lytic enzymes was higher in the mutants than in wild-type cells (Table 2). Perhaps related to cell wall alterations, we observed that, when mutant strains were grown in liquid CM at pH 7, the culture medium became highly viscous after a few days. It was presumed that viscosity was related to secretion of a polysaccharide to the medium. Accordingly, the culture medium was treated with cold ethanol, and neutral sugars were measured in the precipitate formed. It was observed that the BMA2 Δ*rim101* mutant secreted about 8 times more polysaccharide than did the FB2 strain after 48 h and 9.5 times more after 72 h of incubation (Fig. 5A). Interestingly, polysaccharide secretion was pH dependent (Fig. 5B).

(iv) Mating. We analyzed mating of mutant strains BMA2 (*a2b2* Δ*rim101*::*hyg*) and BMA7 (*a1b1* Δ*rim101*::*hyg*) and wild-type strains FB2 (*a2b2*) and FB1 (*a1b1*) in all the different possible combinations. It was observed that all mixtures of sexually compatible strains developed a positive Fuz reaction, independently of their *RIM101* gene status (not shown).

(v) Virulence and development in maize plants. Virulence of Δ*rim101* strains was analyzed by inoculation of mixtures of sexually compatible mutants (BMA2 and BMA7) or wild-type strains (FB1 and FB2) in maize seedlings. No qualitative or quantitative differences in virulence symptoms were observed between mutant and wild-type mixtures (Table 3). To analyze whether the normal life cycle of the mutant strains was being completed in the maize plants, teliospores were recovered from the galls formed in the infected plants. Teliospores from mutants or wild-type strains showed no morphological difference, and when inoculated on solid complete medium, they displayed no differences in their germination capacity (not shown), suggesting that they had no defects in meiosis. It has been reported previously that *Saccharomyces cerevisiae rim101* mutants have alterations in meiosis (43).

(vi) Protease secretion. Secretion of alkaline proteases was analyzed in agar plates containing skim milk as the sole nitrogen source at pH 6.8 or 8.0. After 5 days, wild-type strains developed clear halos of proteolysis on the plates, whereas none of the Δ*rim101* mutants analyzed showed this capacity (Fig. 6A).

Complementation of the *RIM101* mutation. The BMA2 strain was transformed with pUpacC22 or pUpacC23 carrying respectively the truncated or the complete versions of the *RIM101* ORF (see Materials and Methods). Carboxin-resistant clones were recovered, and their resistance to carboxin and hygromycin was confirmed. For further studies, we selected one strain obtained by transformation with pUpacC22 (AAP1) and two (PGM1 and PGM2) obtained after transformation with pUpacC23. AAP1 and PGM1 transformants recovered the capacity to secrete the alkaline protease comparable to FB2 (Fig. 6A). Other phenotypic characteristics of the wild-type strains that had been altered by *RIM101* disruption were

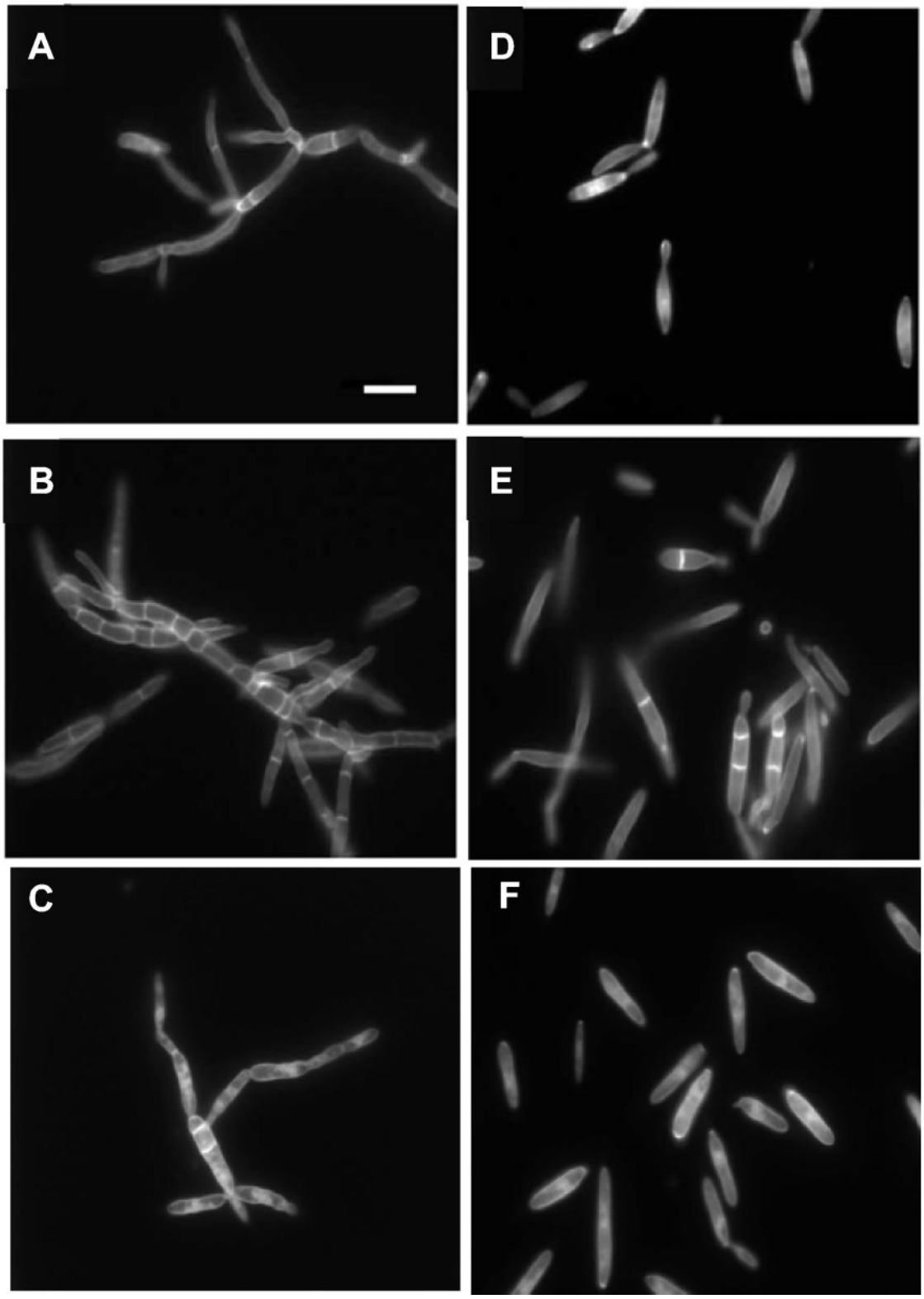

FIG. 4. Cell morphology of *U. maydis* wild-type and mutant strains. Strains were grown in MM at pH 3 (A, B, and C) or pH 7 (D, E, and F) and stained with Calcofluor white. A and D, FB2 (wild type); B and E, BMA2 (Δ*rim101*); C and F, PGM1 (Δ*rim101*::*hyg*/pUpacC23). Bar, 10 μm.

TABLE 2. Several phenotypic characteristics of wild-type, Δ*rim101* mutant, and complemented strains of *U. maydis*[a]

Strain	Yeast form length (μm)	Septa in mycelium[b]	Sensitivity to lytic enzymes[c]
FB2 (wild type)	12.7 ± 1.30	2.40 ± 0.99	54.16 ± 4.79
BMA2 (Δ*rim101*)	17.2 ± 3.50	5.00 ± 0.79	93.25 ± 0.95
PGM1 (Δ*rim101*/pUpacC23)	10.51 ± 2.11	1.92 ± 0.66	39.60 ± 3.60

[a] Differences were found to be significant (see text).
[b] Per 30 μm of length.
[c] As percentage of protoplasts formed after 1 h of treatment with *Trichoderma* lytic enzymes.

TABLE 3. Effect of *RIM101* deletion on virulence of *U. maydis* on maize seedlings

No. of cells inoculated	Plants with galls (%)[a]	
	FB2 × FB1 (wild type)	BMA2 × BMA7 (Δ*rim101*)
10^5	100	100
10^4	72	84
10^3	50	54
10^2	50	31

[a] Six-day-old *Zea mays* seedlings (25 per condition) were inoculated with different cell densities of mixtures of mating-compatible *U. maydis* strains. After 15 days, disease symptoms were noted.

also recovered in strain PGM1. Accordingly, its sensitivity to *Trichoderma* lytic enzymes was reduced (Table 2). Also polysaccharide secretion was restrained: the PGM1 strain produced 0.500 ± 0.03 mg/ml and PGM2 produced 0.630 ± 0.05 mg/ml, after 48 h of growth in CM, pH 7, compared to 0.567 ± 0.04 mg/ml synthesized by the wild-type strain FB2. Tolerance to Li^+ and Na^+ salts was also recovered in PGM1 (Fig. 3), and the cell shape of the complemented strain PGM1 was very similar to that of the wild type at either pH 3 or pH 7 (Table 2; Fig. 4C and F).

Complementation of a null *rim101 Y. lipolytica* mutant with *U. maydis RIM101*. *Y. lipolytica* strain FL3 (*A rim101-1113 ura3 lys11*) was transformed with the autonomous replication plasmid pUpacC44 bearing the truncated form of the *U. maydis RIM101* ORF under the control of the promoter and terminator of the *Y. lipolytica Xpr2* gene. Transformants were isolated in uracil-free medium, and their uracil prototrophy was confirmed by transfer to fresh plates of the same medium. One hundred transformants were inoculated on skim milk plates at pH 6.8, and we observed that about 70% of the isolates recovered alkaline protease activity (not shown). Figure 6B shows proteolysis in skim milk plates by one transformant (strain ETA1) compared to *Y. lipolytica* wild-type strain E121 and contrasting with FL3ΔR (*rim101* mutant). Five strains recovered after transformation with the empty plasmid pINA444 carrying *Ura3*, but not the *RIM101* gene, recovered uracil prototrophy but not protease activity (not shown).

DISCUSSION

Signaling by pH in fungi is an important mechanism for their survival in nature. At least three mechanisms have been described to fulfill this function. One of the best-characterized pH-transduction systems is the Pal/Rim pathway (reviewed in

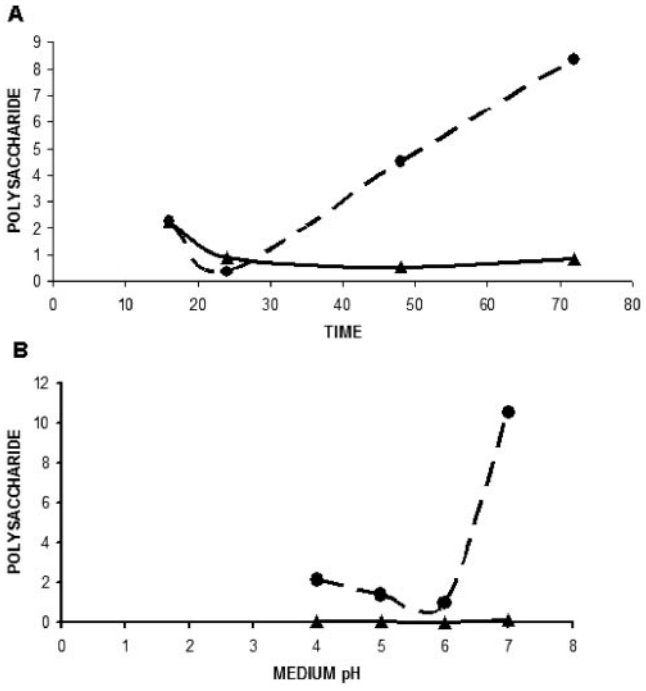

FIG. 5. Polysaccharide secretion by wild-type and mutant strains of *U. maydis*. A. Strains FB2 and BMA2 were grown in pH 7 CM. At different times, the polysaccharide was precipitated with ethanol, and the content of neutral sugars was measured. B. Cells were grown for 48 h in MM of different pH values. Triangles and solid lines, strain FB2; circles and broken lines, strain BMA2. Polysaccharide data are expressed in mg/ml.

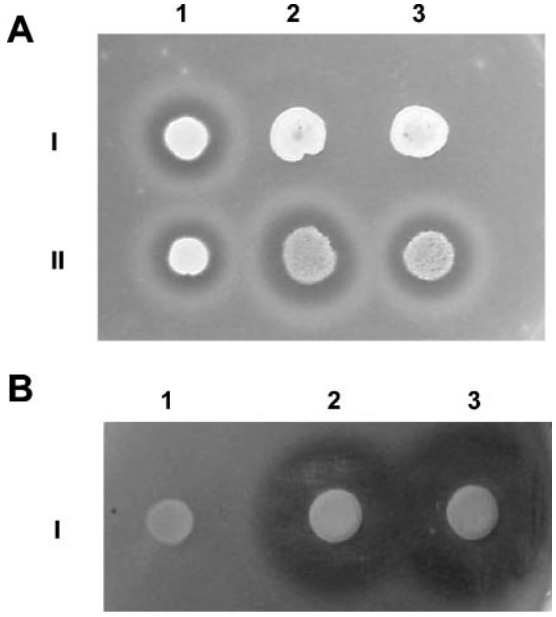

FIG. 6. Proteolytic activities of different *U. maydis* and *Y. lipolytica* strains. Strains were inoculated over skim milk plates and incubated for 48 h. A. *U. maydis*. I/1, FB2 wild-type strain; II/1, FB1 wild-type strain; I/2, BMA2 Δ*rim101* mutant; I/3, BMA4 Δ*rim101* mutant; II/2, PGM1 complemented strain; II/3, AAP1 complemented strain. B. *Y. lipolytica*. I/1, ΔFL3 mutant; I/2, ETA1 complemented strain; I/3, E121 wild-type strain.

references 32 and 33). A mechanism responsive to alkaline pH dependent on calcineurin that acts by activation of a large number of genes through the transcriptional factor Crz1p has been described elsewhere for yeast (41, 42; see reviews in references 32 and 33). Finally a mechanism depending on the *MDS3* gene was recently described for *C. albicans*. *MDS3* encodes a polypeptide possessing a kelch-like interaction domain. Homozygous *mds3/mds3 C. albicans* mutants were affected in the expression of several genes related to mycelial growth, failed to form mycelium at alkaline pH in some media, and displayed reduced virulence in a model system (14). Apparently this protein acts in parallel with Rim101p (14; reviewed in reference 33).

In the present study we analyzed whether the Pal/Rim pathway could be involved in the control of dimorphism of *U. maydis*, which depends on external pH (38). Accordingly, we proceeded to determine if a homologue of the *RIM101/pacC* gene was present in *U. maydis* (all previously described homologues belong to ascomycetes and deuteromycetes) and, if so, to isolate the encoding gene. The homologue of *U. maydis RIM101/pacC* (named *RIM101*) identified in this study encodes a protein with highest similarity to PacC proteins from *F. oxysporum* and *A. niger*. As for other reported homologues, maximal similarity is concentrated at the zinc finger-encoding regions and reduced at the C termini of the proteins (24).

The expression of *RIM101* measured by Northern blotting showed much higher values at neutral pH. Hygromycin resistance of disruptants (a qualitative assay in strains where the hygromycin resistance gene was under the control of the *RIM101* promoter) was found also at both pH 3 and pH 7. It is known that, in different fungi, such as *C. albicans* (13), *Y. lipolytica* (24), *A. niger* (26), and *Sclerotinia sclerotiorum* (36), PacC/Rim101 mRNA is present at all physiological pH values, but PacC/Rim101 stimulates its own transcription only at neutral or alkaline pH. If the two putative PacC/Rim101 binding boxes detected in the promoter region of *U. maydis RIM101* are functional, this might explain the difference in expression observed at both pH values.

U. maydis RIM101 was identified as the homologue of *pacC/RIM101* by two pieces of evidence: (i) from a structural point of view, by the sequence homology of Rim101p to other Pal/Rim101 proteins, and (ii) from a functional point of view, by its capacity to complement the alkaline protease activity in a *Y. lipolytica* Δ*rim101* mutant. Activity of PacC/Rim101 as a transcription factor in other systems (e.g., references 25, 26, and 44) requires its proteolytic processing. In case processing might be different in *U. maydis* and *Y. lipolytica*, a partial version of the *RIM101* ORF that included the zinc finger coding region was used for transformation. This version (first demonstrated to be functional for the complementation of the alkaline protease secretion in *U. maydis* Δ*rim101* mutants) proved to be effective in restoring the proteolytic activity in the *Y. lipolytica* Δ*rim101* mutant.

Disruption of *RIM101* by double homologous recombination did not affect the growth rate of *U. maydis* mutants at acid or neutral pH, suggesting that, as in other systems (13, 25, 26, 36), the gene is dispensable for growth. Also, mutants were not affected in their dimorphic capacity, a result suggesting that, in contrast to *C. albicans*, in which *RIM101* is required for pH-dependent filamentation (34), the Pal/Rim pathway is not involved in the pH signal transduction required for dimorphism in *U. maydis*. Another contrast between *U. maydis* and *C. albicans* mutants regards virulence. It has been reported previously that *C. albicans RIM101* is involved in persistence of infection (11); in contrast Δ*rim101* mutants of *U. maydis* were as virulent in maize as were wild-type strains. Apparently this behavior depends on the fungal species. *Fusarium oxysporum pacC* mutants are more virulent than the wild-type strains, suggesting that PacC behaves as a negative regulator of virulence (9). In contrast, *S. sclerotiorum pacC* mutants display reduced virulence (35).

Nevertheless, as occurs with other fungi (9, 23; reviewed in references 32 and 33), *U. maydis* mutants deficient in the Rim101-encoding gene displayed a pleiotropic phenotype. Δ*rim101* mutants were abnormal regarding protein secretion, cell morphogenesis, cell wall strength, polysaccharide secretion, and Na^+ and Li^+ stress tolerance. All these alterations were alleviated by transformation with a plasmid carrying the wild-type version of the *RIM101* gene. These results are evidence that the *RIM101* mutation was directly responsible for all those phenotypic alterations.

One of the initially observed characteristics of fungal mutants deficient in genes involved in the Pal/Rim pathway was their impaired capacity to secrete different enzymes in response to changes in pH (reviewed in references 32 and 33). In agreement with this behavior, *U. maydis*Δ*rim101* mutants were deficient in extracellular protease activity. They were also altered in morphogenesis: mutant yeast cells were larger, and occasionally septated, a rare event in wild-type yeast cells. Frequency of septation in mutant mycelial cells grown at acid pH was increased. These alterations suggest that Rim101 might be involved in morphogenesis and the regulation of the cell cycle. In agreement with this hypothesis, it has been reported that Rim101/PacC is involved in different morphogenetic phenomena in other fungi. Accordingly, evidence exists that that the gene product plays a role in the regulation of sporulation and invasive growth of *S. cerevisiae* (25, 43), efficient conidiation of *A. nidulans* (44), and sclerotial development in *S. sclerotiorum* (35). Morphogenetic alterations of *U. maydis* occurring in the mycelium of mutants deficient in *RIM101* growing at pH 3 suggest that the gene product has an active function at acid pH. The observations that *A. nidulans pacC* mutants display reduced conidiation and abnormal morphology at acid pH (44) and that chlamydospore formation in medium at pH 5.5 decreases in *rim13* and *rim101* mutants of *C. albicans* (29) agree with our results and reinforce this hypothesis. For *S. cerevisiae* it was reported (25) that the processed (and presumptively active) form of Rim101 protein was present at both alkaline and acid pH.

In regard to other phenotypic alterations of Δ*rim101* mutants, we may hypothesize that their increased sensitivity to lytic enzymes (an indication of alterations in the structure of the cell wall) and their increased secretion of polysaccharide are related phenomena. In *C. albicans*, PacC/Rim101 has been related to the regulation of Phr1 and Phr2 surface proteins. These are retained in the plasmalemma through a glycosylphosphatidylinositol anchor and possess a transglycosidase activity that may play a role in the linking of β-1,6 and β-1,3 glucans in the cell wall (17, 40). A similar role for other members of the same transglycosidase family in different fungi has

been suggested (for a discussion see reference 37). A plausible hypothesis is that a similar mechanism operates in *U. maydis* and that mutation of *RIM101* alters the correct functioning of putative transglycosidases. As a result, a polysaccharide involved in cell wall growth would be secreted (at least partially) to the medium, instead of being bound to the wall, thus resulting in its abnormal structure and organization.

Another phenotypic alteration brought about by the *RIM101* mutation was an elevated susceptibility to Na^+ and Li^+, but not K^+, ions. A similar phenomenon was reported for *pacC*/*rim101* mutants of *F. oxysporum* and *S. cerevisiae*. This sensitivity appears to be due to the possible alteration in the expression of a P-type Na^+-ATPase, Ena1 (9, 23). Interestingly, in *S. cerevisiae* a plasma membrane-bound ENA1-type Na^+-ATPase was up-regulated by the calcineurin/Crz1 pathway cited above (41). In *U. maydis* two novel P-type ATPases that mediate K^+ or Na^+ uptake have been isolated, but these are not related to Ena1 ATPases (6). Also the observation that their transcripts accumulated at acid pH (6) makes improbable their regulation by Rim101, since according to our results sensitivity to salt stress occurred only at alkaline pH. The observation that high concentrations of sorbitol did not affect *U. maydis* growth is evidence (as suggested in reference 9) of a specific sensitivity to salt and not to hyperosmotic stress.

Originally, the role of the Pal/Rim pathway was considered to be specific to ascomycetes and deuteromycetes and involved in the expression of some selected pH-regulated exoenzymes. Further data have revealed that the pathway plays unsuspected important roles in other cellular functions (9, 23, 35). The results described here are evidence that the pathway also exists in basidiomycetes, having roles in a series of similar complex phenomena with several heretofore-unobserved phenotypic consequences. In this sense it is relevant that an in silico analysis of the *U. maydis* genome revealed the existence of putative homologues of genes *palA*/*RIM20*, *palB*/*RIM13*, and *palI*/*RIM9*.

ACKNOWLEDGMENTS

This work was partially supported by CONACYT, México. The doctoral studies of E.T.A.-C. were supported by a CONACYT fellowship.

We thank Flora Banuett for providing us *U. maydis* strains FB1 and FB2, Claude Gaillardin for *Y. lipolytica* strains FL3ΔR and E121, Scott Gold for valuable comments on this work, Peter Schrier for the partial *RIM101* sequences provided, and Claudia León for her valuable technical support.

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