Dominant Active Alleles of *RIM101* (*PRR2*) Bypass the pH Restriction on Filamentation of *Candida albicans*

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Morphological development of the fungal pathogen *Candida albicans* is profoundly affected by ambient pH. Acidic pH restricts growth to the yeast form, whereas neutral pH permits development of the filamentous form. Superimposed on the pH restriction is a temperature requirement of approximately 37°C for filamentation. The role of pH in development was investigated by selecting revertants of *phr2Δ* mutants that had gained the ability to grow at acid pH. The extragenic suppressors in two independent revertants were identified as nonsense mutations in the pH response regulator *RIM101* (*PRR2*) that resulted in a carboxy-terminal truncation of the open reading frame. These dominant active alleles conferred the ability to filament at acidic pH, to express *PHR1*, an alkaline-expressed gene, at acidic pH, and to repress the acid-expressed gene *PHR2*. It was also observed that both the wild-type and mutant alleles could act as multicopy suppressors of the temperature restriction on filamentation, allowing extensive filamentation at 29°C. The ability of the activated alleles to promote filamentation was dependent upon the developmental regulator *EFG1*. The results suggest that *RIM101* is responsible for the pH dependence of hyphal development.

Candida albicans is the predominant fungal pathogen of humans (13). It is commonly associated with the gut of humans as well as other warm-blooded animals and is considered an obligate saprophyte (8, 24). The most important reservoir of *C. albicans* in human disease is believed to be endogenous, and this pathogen can infect most tissues and organs, indicating that it is well adapted for survival within the diverse environmental niches of its host (24). *C. albicans* is polymorphic, able to change reversibly between round budding yeast and elongated hyphae or filamentous growth forms. This morphological flexibility appears to be a key contributor to virulence (21). The yeast form predominates under standard culture conditions, but hyphal development occurs in diverse host niches and can be promoted in vitro by diverse culture conditions.

Among the environmental variables that influence filamentation in vivo, ambient pH has a defining role (1, 4, 10). Optimal filamentation occurs near neutral pH and is much reduced at pH below 6.0. The yeast form is exclusively present at pH 4.0 (4). In conjunction with neutral pH, filamentation is favored by an elevated temperature of around 37°C and is largely absent below 34°C (4).

The molecular mechanisms that govern the relationship between environment and morphological development are not clear. *CPH1* and *EFG1* encode two transcription factors important in this process. *CPH1* appears to lie at the end of a mitogen-activated protein kinase cascade analogous to *STE12* of *Saccharomyces cerevisiae* (20). *EFG1* lies downstream of *TPK2*, a cyclic AMP (cAMP)-dependent protein kinase required for filamentation (3, 35). The environmental signals that activate these two pathways are unknown.

The pH response depends upon a zinc finger transcription factor, *RIM101/PRR2*, hereafter referred to as *RIM101*, which is related to *PacC* of *Aspergillus nidulans* and *RIM101* of *S. cerevisiae* (6, 19, 27, 39, 40). Mutants lacking *RIM101* are defective in filamentation and pH-dependent gene regulation (6, 27, 40). *C. albicans* responds to changes in environmental pH by differential expression of several genes, including *PHR1* and *PHR2* (23, 29, 31). *C. albicans* can be confronted by the neutral pH of blood during the course of a generalized infection or the acidic pH of the vagina and skin (24). Differential, pH-dependent expression of *PHR1* and *PHR2* is not restricted to in vitro conditions but appears to be similarly controlled by the pH of the host niche (7). *PHR2* is an acid-expressed gene that is not expressed at detectable levels above pH 6.5. Mutants lacking *PHR2* are unable to grow at acidic pH and exhibit morphological defects (23). *PHR1* is an alkaline-expressed gene with the inverse pattern of expression. *PHR1* and *PHR2* encode functionally homologous proteins involved in cell wall biosynthesis, which is pivotal in cell shape changes during dimorphism (11, 22, 23, 29). pH-regulated dimorphism and pH-dependent differential gene expression are also observed in the polymorphic fungal pathogen *Candida dubliniensis*, which is closely related to *C. albicans* (16, 30).

In this report, further insight into the roles of *PHR1*, *PHR2*, and the pH response in morphogenesis was sought by reversion analysis of a homozygous *phr2Δ* mutant. The revertants were selected by restoration of growth at acidic pH. The phenotype of revertants included pH-independent expression of *PHR1* and the ability to filament at acidic pH. Detailed analysis of two revertants demonstrated that they had acquired dominant activating mutations in *RIM101*. Unexpectedly, *RIM101* was found to act as a multicopy suppressor of the temperature requirement for filamentation. In addition, we show that pH-regulated dimorphism but not pH-dependent gene expression requires the transcription factor Efg1p.

MATERIALS AND METHODS

Strains and growth conditions. The *C. albicans* strains used in this study are listed in Table 1. YPD and YNB media were prepared as described before (33).

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TABLE 1. *C. albicans* strains used in this study

Strain	Parent	Genotype	Reference
SC5314		Clinical isolate	15
CAF3-1		*ura3Δ::λimm434/ura3Δ::λimm434*	12
CFM-2		*phr2Δ::hisG/phr2Δ::hisG-URA3-hisG ura3Δ::λimm434/ura3Δ::λimm434*	23
CFM-4		*phr2Δ::hisG/phr2Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434*	23
CFM-5		*phr2Δ::hisG/phr2Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434-(pBSK-TEF1$_{pr}$::PHR1-URA3)*	23
CEM-1	CFM-4	*RIM101-1426/RIM101 phr2Δ::hisG/phr2Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434*	This work
CEM-2	CFM-2	*RIM101-1751/RIM101 phr2Δ::hisG/phr2Δ::hisG-URA3-hisG ura3Δ::λimm434/ura3Δ::λimm434*	This work
CEM-5	CEM-1	*RIM101-1426/rim101Δ::hisG-URA3-hisG phr2Δ::hisG/phr2Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434*	This work
CEM-5U	CEM-5	*RIM101-1426/rim101Δ::hisG phr2Δ::hisG/phr2Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434*	This work
CEM-6	CEM-1	*rim101-1426Δ::hisG-URA3-hisG/RIM101 phr2Δ::hisG/phr2Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434*	This work
CEM-7	CEM-1	*ura3Δ::λimm434/ura3Δ::λimm434-(pBSK+ -RIM101-1426-URA3) RIM101-1426/RIM101 phr2Δ::hisG/phr2Δ::hisG*	This work
CEM-8	CEM-1	*ura3Δ::λimm434/ura3Δ::λimm434-(pBSK+ -RIM101-1426-URA3)$_{n≥2}$ RIM101-1426/RIM101 phr2Δ::hisG/phr2Δ::hisG*	This work
CEM-10	CEM-5U	*ura3Δ::λimm434/ura3Δ::λimm434-(pBSK+ -RIM101-1426-URA3) RIM101-1426/rim101Δ::hisG phr2Δ::hisG/phr2Δ::hisG*	This work
CEM-16-1	CFM-4	*ura3Δ::λimm434/ura3Δ::λimm434-(pBSK+ -RIM101-1426-URA3) RIM101/RIM101 phr2Δ::hisG/phr2Δ::hisG*	This work
CEM-31-1	CFM-4	*ura3Δ::λimm434/ura3Δ::λimm434-(pBSK+ -RIM101-URA3)RIM101/RIM101 phr2Δ::hisG/phr2Δ::hisG*	This work
CEM-16-3	CFM-4	*ura3Δ::λimm434/ura3Δ::λimm434-(pBSK+ -RIM101-1751-URA3) RIM101/RIM101 phr2Δ::hisG/phr2Δ::hisG*	This work
CAF3-16-1	CAF3-1	*ura3Δ::λimm434/ura3Δ::λimm434-(pBSK+ -RIM101-1426-URA3) RIM101/RIM101*	This work
CAF3-31-1	CAF3-1	*ura3Δ::λimm434/ura3Δ::λimm434-(pBSK+ -RIM101-URA3) RIM101/RIM101*	This work
JKC18		*cph1Δ::hisG/cph1Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434*	20
JKC18-pSM2	JKC18	*cph1Δ::hisG/cph1Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434-(pBSK+ -URA3) RIM101/RIM101*	This work
JKC18-16	JKC18	*cph1Δ::hisG/cph1Δ::hisG/cph1Δ::hisG ura3Δ::λimm434 (-pBSK+ -RIM101-1426-URA3) RIM101/RIM101*	This work
JKC18-31	JKC18	*cph1Δ::hisG/cph1Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434 -(pBSK+ -RIM101-URA3) RIM101/RIM101*	This work
HLC67		*efg1Δ::hisG/efg1Δ::hisG ura3Δ:: λimm434/ura3Δ::λimm434 RIM101/RIM101*	21
HLC67-pSM2	HLC67	*efg1Δ::hisG/efg1Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434 -(pBSK+ -URA3) RIM101/RIM101*	This work
HLC67-16	HLC67	*efg1Δ::hisG/efg1Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434 −(pBSK+ -RIM101-1426-URA3) RIM101/RIM101*	This work
HLC67-31	HLC67	*efg1Δ::hisG/efg1Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434 −(pBSK+ -RIM101-URAe) RIM101/RIM101*	This work
CDB1		*cph1Δ::hisG/cph1Δ::hisG efg1Δ::hisG/efg1Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434 RIM101/RIM101*	21; Bockmühl, unpublished data
CDB1-pSM2	CDB1	*cph1Δ::hisG/cph1Δ::hisG efg1Δ::hisG/efg1Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434-(pBSK+ -URA3) RIM101/RIM101*	This work
CDB1-16	CDB1	*cph1Δ::hisG/cph1Δ::hisG efg1Δ::hisG/efg1Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434-(pBSK+-RIM101-1426-URA3) RIM101/RIM101*	This work
CDB1-31	CDB1	*cph1Δ::hisG/cph1Δ::hisG efg1Δ::hisG/efg1Δ::hisG ura3Δ::λimm434/ura3Δ::λimm434-(pBSK+ -RIM101-URA3) RIM101/RIM101*	This work

Medium 199 was prepared as previously described (23), and the medium of Lee et al. was prepared as described before (18). Media were supplemented with uridine (25 mg/ml) as needed. 5′-Fluoroorotic acid-containing medium was prepared as described by Boeke et al. (2), except that uridine was substituted for uracil. Media were solidified with 2% agar. To test the effect of acid culture conditions on filamentation in liquid medium, cells were cultured overnight to the stationary phase at 28 or 37°C in medium 199, YNB, or Lee's medium, each adjusted to pH 4.0. The stationary-phase cells were inoculated into fresh medium of the same composition at a density of 5×10^6 cells/ml and incubated at 29 or 37°C for 4 h on a rotary shaker. Filamentation on agar-solidified medium was assessed using medium 199 (pH 4.0). The plates were spotted with 10^6 cells in 5 μl of sterile water and incubated at 37°C for 3 to 6 days.

Isolation of *phr2Δ* revertants. Strain CFM-4 (*phr2Δ ura3Δ*) (23) was grown to stationary phase in YPD (pH 7.0). After washing with sterile distilled H_2O, 10^8 cells were spread on each of five YNB agar plates adjusted to pH 4.0 and containing 25 μg of uridine per ml. The plates were incubated for 3 days at 30°C. Colonies growing at this restrictive pH were recovered with a median frequency

of approximately 1.5×10^{-7}. One of the CFM-4 revertants, named CEM-1, was chosen for further analysis. A second revertant, designated CEM-2, was derived from strain CFM-2 (*phr2Δ URA3*) (23).

Disruption of *RIM101* in CEM-1. Plasmid pARA3 (27) was used to delete one or the other of the two *RIM101* alleles in CEM-1. Plasmid DNA was digested with *Hin*dIII and *Ssp*BI to release a 5-kb *RIM101* disruption construct (27), and 8 μg of the gel-purified fragment was used to transform CEM-1 to Uri+ using lithium acetate (14). Transformants were selected on YNB buffered to pH 7.0 with 150 mM HEPES. The resulting colonies were replica plated in parallel to YNB adjusted to either pH 4.0 or 7.0 and assessed for growth following incubation for 2 days at 30°C. Transformants that retained the CEM-1 phenotype, growth at both pH 4 and pH 7, were designated type-1 transformants. CEM-5 is a representative type-1 transformant. Transformants that had lost the ability to grow at pH 4.0 were classified as type-2 transformants, as represented by strain CEM-6. Integration of the transforming DNA at the *RIM101* locus was confirmed by Southern blotting.

Cloning and sequencing of *RIM101* alleles from the revertants. *RIM101* was amplified by PCR using oligonucleotide primers PRR2-3 (5′-ACGACCTTATA TGCGTAATCC-3′) and PRR2-4 (5′-GAACCATGTAAATAGAGAACGG-3′). The primers are located 753 nucleotides 5′ and 181 nucleotides 3′, respectively, of the 1,986-bp *RIM101* coding region. Amplification was performed with an initial denaturation step of 95°C for 3 min, followed by 15 cycles, each cycle consisting of 40 s at 95°C, 1 min at 58°C, and 2 min at 72°C and a final step of 10 min at 72°C. When 1 μg of genomic DNA from type-1 and type-2 transformants was used as the template, two amplification products of 2.9 and 5 kb were obtained. These corresponded to the functional and disrupted alleles, respectively. The 2.9-kb product from two independent type-1 transformants was cloned into vector pCR2.1 (Invitrogen) to generate plasmids pCR-16-1 and pCR-16-2. Analogous cloning from two independent type-2 transformants yielded plasmids pCR-31-1 and pCR-31-2. The inserts from all four plasmids were sequenced on both strands. Four additional independent clones were generated and partially sequenced to verify sequence differences.

In analyzing the *RIM101* locus of revertant CEM-2, both alleles were amplified as a mixed PCR product using primers PRR2-3 and PRR2-4, 1 μg of genomic DNA as the template, and 15 cycles of amplification. The resulting 2.9-kb PCR product was gel purified and cloned into vector pCR2.1. The PCR product was sequenced directly using primer PRR2-11 (5′-CCTCAACAGCAACACCCAC-3′) or following cloning into pCR2.1. Sequence analysis identified two cloned alleles, the wild type, represented by plasmid pCR-31-3, and a mutant allele, represented by plasmid pCR-16-3.

Introduction of recovered *RIM101* alleles by transformation. The *RIM101* alleles recovered from the revertants were introduced into various *C. albicans* strains by transformation. The inserts from plasmids pCR-16-1, pCR-16-3, pCR-31-1, and pCR-31-3 were released by XbaI-SpeI digestion and cloned into the XbaI site of plasmid pSM-2. Plasmid pSM-2 consists of a 3.85-kb XbaI fragment containing *URA3* blunt-end ligated into the SmaI site of pBSK(+) (Stratagene). The resulting plasmids, pEM-16-1, pEM-16-3, pEM-31-1, and pEM-31-3, respectively, were made linear by digestion at the unique HpaI site adjacent to *URA3* prior to transformation. Uri⁺ transformants were selected, and proper targeting of the plasmid was confirmed by Southern blot analysis. The transformed strains included CFM-4 (*phr2Δ ura3Δ*), CAF3-1 (*ura3Δ*), CEM-1 (*ura3Δ RIM101/ RIM101-1426*), CEM-5U (*ura3Δ rim101/RIM101-1426*), JKC18 (*ura3Δ cph1Δ*), HLC67 (*ura3Δ efg1Δ*), and CDB1 (*ura3Δ cph1Δ efg1Δ*). CDB1 (21; D. P. Bockmühl, unpublished data), and CEM-5U are Uri⁻ derivatives of HLC54 and CEM-5A (*rim101Δ/RIM101-1426*), respectively, obtained by selection on medium containing 5-fluoroorotic acid. All transformation events were confirmed by Southern blot analysis.

Southern and Northern blot analysis. Hybridization of Southern and Northern blots was conducted as previously described (23; Bockmühl, unpublished). Hybridization probes used to detect *PHR1*, *PHR2*, and *RIM101* have been described previously (23, 27, 29). For hybridization with *HWP1*, a PCR product amplified with nucleotide primers HWP1-1 (5′-ATGAGATTATCAACTGCTC AA-3′), HWP1-2 (5′-TTAGATCAAGAATGCAGCAAT-3′), and genomic DNA from strain SC5314 as the template, was used (36). Hybridization with *ACT1* was conducted with a PCR amplification product generated with primers OK1 (5′-TGTTTTCCCATCCCTCGT-3′) and OK2 (5′-TTCGTCGTATTCTT GTTT-3′) and genomic DNA from SC5314 as the template. Both probes generated by PCR were partially sequenced prior to use to verify the presence of the desired amplification product.

RESULTS

Spontaneous revertants of *phr2Δ* mutants are altered in growth and filamentation. Following a shift from a permissive pH of 7.0 to a restrictive pH of 4.0, *phr2Δ* mutants undergo one mass doubling and then cease growth (23). This phenotype allowed easy selection of revertants in which growth was restored at the restrictive pH (Fig. 1b). Spontaneous revertants arose at a median frequency of 1.5×10^{-7}. In addition to the growth defect, *phr2Δ* mutants incubated at the restrictive pH form enlarged, morphologically abnormal cells due to defective cell wall assembly (11, 23). This aberrant morphology was no longer evident upon microscopic examination of cells of the revertant, suggesting that, in addition to the growth defect, the defect in cell wall biosynthesis was also suppressed. An unexpected phenotype associated with the revertants was the ability to filament at acidic pH. It was noted that the colonies of the revertants on either neutral or acidic medium exhibited an atypical rough colony morphology, which has been associated with the presence of hyphae and pseudohyphae (20). Microscopic examination of cells from these colonies verified that they were composed of a mixture of cell forms, including

yeasts, hyphae, and pseudohyphae. This observation was surprising because acidic pH suppresses filamentation of wild-type *C. albicans*. Therefore, the effect of pH on the ability of the revertant strains to initiate hyphal development in the form of germ tubes was examined. When tested in medium 199 (pH 7.5) at 37°C, conditions conducive to germ tube formation, there was no notable difference between the wild-type control strain, SC5314, and the revertant, CEM-1. The percent germination in three independent experiments averaged 96% ± 1% for SC5314 and 88% ± 6% for CEM-1. However, a dramatic difference was observed when the medium was adjusted to pH 4.0. Less than 1% of the wild-type cells formed germ tubes, but germination of CEM-1 or CEM-2 was comparable to that at neutral pH, 88% ± 1% (Fig. 1c; see Fig. 4b; and data not shown). Similar frequencies of germ tube formation at pH 4 were observed with the revertant CEM-2 and when alternative media were used, including the medium of Lee et al. and YNB. Additional strain controls included the *phr2Δ* parents of the revertants, CFM-2 and CFM-4, and strain CAF3-1, from which CFM-2 and CFM-4 were derived. At pH 4.0 CFM-2 and CFM-4 exhibited the previously reported abnormalities (23) and strain CAF3-1 failed to form germ tubes, indicating that the phenotype was not due to extraneous mutations introduced in the construction of CFM-2 and CFM-4 (Fig. 1c). The ability of the revertants to filament at acidic pH was not restricted to liquid suspension culture but was also observed on agar-solidified medium 199 and YNB (data not shown).

PHR1 expression is no longer pH dependent in the revertants. Previous studies demonstrated that Phr1p and Phr2p are functionally analogous (23). Since *PHR1* is normally expressed only at a pH of ≥5.5, its ability to complement a *phr2Δ* mutation was demonstrated by using the constitutive promoter from *TEF1* to drive *PHR1* expression at acidic pH (23). Expression of *PHR1* in this manner complemented both the growth and morphological defects of the *phr2Δ* mutant. Based on these observations, we asked whether the pH dependence of *PHR1* expression was altered in the spontaneous revertants. The *phr2Δ* mutant exhibited the expected pH-dependent pattern of *PHR1* expression, as assessed by Northern blot analysis (Fig. 1d). In contrast, expression of *PHR1* in the revertant strains CEM-1 and CEM-2, as well as three other independent revertants, was unaffected by the pH of the culture medium; comparable levels of mRNA were present in cells cultured both at neutral and at acidic pH (Fig. 1d and data not shown).

The constitutive expression of *PHR1* in the revertants could account for the restoration of growth and cell morphology, but it was not known if this could account for the ability of the revertants to filament at acidic pH. To address this issue, strain CFM-5, in which *PHR1* is constitutively expressed from the *TEF1* promoter (23), was examined for its ability to filament at pH 4.0. No filamentation was observed (Fig. 1c) even though the steady-state levels of *PHR1* mRNA in CFM-5 were similar to that in the revertants (Fig. 1d). Thus, the acidic filamentation phenotype of the revertants was not directly related to the pH-independent expression of *PHR1*.

Evidence of heterozygosity at the *RIM101* locus of the revertants. The foregoing suggested either that the revertants contained a single mutation that simultaneously affected *PHR1* expression and filamentation or that the revertants had acquired a minimum of two mutations, one specific to each of these phenotypes. Two pieces of evidence suggested that the revertants had acquired a single dominant mutation. The frequency of revertants was much higher than expected for a spontaneous homozygous recessive mutation but possibly lower than expected for other events that might uncover an existing recessive mutation. Furthermore, low-dose UV treat-
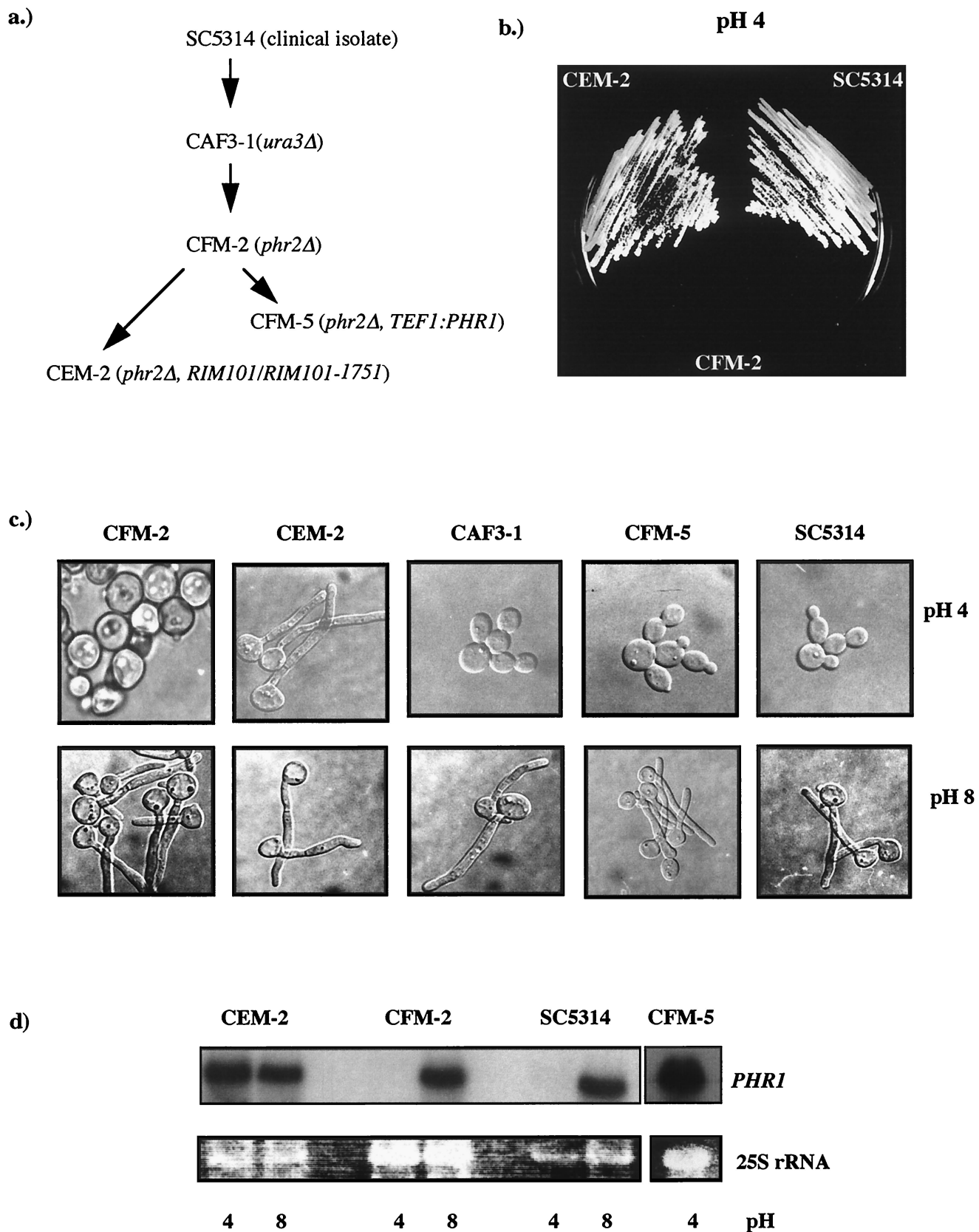
a.)

SC5314 (clinical isolate)

↓

CAF3-1(*ura3Δ*)

↓

CFM-2 (*phr2Δ*)

↙ ↘

CFM-5 (*phr2Δ, TEF1:PHR1*)

CEM-2 (*phr2Δ, RIM101/RIM101-1751*)

b.) pH 4

CEM-2   SC5314

CFM-2

c.)

CFM-2	CEM-2	CAF3-1	CFM-5	SC5314	
					pH 4
					pH 8

d)

CEM-2   CFM-2   SC5314   CFM-5

PHR1

25S rRNA

4   8   4   8   4   8   4   pH

FIG. 1. Phenotypes of revertant CEM-2. (a) Genealogy of CEM-2. The relevant genotypes are shown. (b) Growth of wild-type strain SC5314, the *phr2Δ* mutant CFM-2, and the revertant CEM-2 after 48 h of incubation on YNB at pH 4.0 and 30°C. (c) Morphology of the indicated strains 3 h postinoculation into medium 199 at pH 4.0 or pH 8 and 37°C. (d) *PHR1* expression as a function of ambient pH. RNA was isolated from the indicated strains cultured in medium 199 at the pH indicated below the panels. The upper panel shows the Northern blot hybridized with *PHR1*. The lower panel shows the corresponding ethidium bromide-stained agarose gel prior to blotting.

ment resulted in a high frequency of back-mutation to the parental phenotype, consistent with a heterozygous dominant mutation (F. A. Mühlschlegel and W. A. Fonzi, unpublished data). These observations were coupled with recent data showing that the pH response regulator *RIM101* controls pH-dependent expression of *PHR1* and *PHR2* and is required for filamentous growth (6, 27). Furthermore, dominant active mutations of the *RIM101* orthologs of *Aspergillus nidulans* and *Yarrowia lipolytica* result in pH-independent expression of alkaline-induced genes (17, 39), analogous to the expression pattern of *PHR1* in the revertants. Thus, we tested the hypothesis that a heterozygous dominant mutation in *RIM101* gave rise to the revertants. This was tested by targeted deletion of one allele of *RIM101*. If in the revertants this locus harbored a heterozygous dominant allele, then disruption of the dominant allele would restore the parental phenotypes, whereas disruption of the wild-type allele would not. These alternate outcomes were expected to occur with roughly similar frequency.

The revertant CEM-1 was transformed with a *hisG-URA3-hisG* cassette replacing 1,270 bp of the *RIM101* coding region, and 40 Uri$^+$ transformants were recovered at pH 7.0 from two independent transformations. Twelve representative isolates were examined by Southern analysis, and all were disrupted at the desired locus, indicating that the majority of transformants resulted from homologous recombination at the *RIM101* locus.

The growth phenotype of the transformants was consistent with the hypothesized heterozygosity. Twenty-five of the 40 transformants were the same as CEM-1, that is, they retained the ability to grow at both acidic and alkaline pH. These were designated type-1 transformants and are represented by strains CEM-5A and CEM-5B (Fig. 2b). The other 15 transformants, however, were unable to grow at pH 4.0, indicating a loss of the revertant phenotype and restoration of the parental growth pattern. This is the phenotype expected for disruption of the dominant allele. These were called type-2 transformants and are represented by strains CEM-6A and CEM-6B (Fig. 2b).

Restoration of the parental growth phenotype was accompanied by the simultaneous restoration of the pH dependence of *PHR1* expression. This was demonstrated with Northern blots of the type-2 strains CEM-6A and CEM-6B, in which *PHR1* mRNA was abundant in alkaline-grown cells and absent in acid-grown cells (Fig. 2c). In contrast, the type-1 transformants CEM-5A and CEM-5B maintained the pH-independent expression pattern characteristic of the revertants. In addition, all 25 type-1 transformants maintained the capacity to filament at pH 4.0, while all 15 type-2 transformants lost this ability (Fig. 2d). Thus, all three phenotypes of the revertants could be simultaneously restored to parental types by disruption of one allele of *RIM101*. These findings were consistent with the hypothesis that the revertants were heterozygous for a dominant active mutation at the *RIM101* locus.

One allele of *RIM101* is mutated in the revertants. Direct evidence for a heterozygous mutation at the *RIM101* locus was obtained by cloning and sequencing both alleles. Because one allele was deleted and genetically tagged in the type-1 and type-2 transformants, the functional allele in these strains could be specifically identified and cloned. The intact allele was amplified by PCR from the type-1 transformants CEM-5A and CEM-5B and the type-2 transformants CEM-6A and CEM-6B. The resulting 2.9-kb fragment encompassed the 1,986-bp open reading frame plus 753 bp 5′ and 184 bp 3′. The allele recovered from CEM-5A and CEM-5B contained a single base substitution, a C-to-T transition at position +1426 of the open reading frame. This mutation lies in the first position of glutamine codon 476 and converts it to a nonsense codon. As a result, the 661-amino-acid protein predicted for the wild-type

gene is truncated by 186 amino acids. Thus, the revertant phenotype exhibited by these type-1 transformants was associated with this mutant allele. In contrast, the product recovered from CEM-6A and CEM-6B was identical in sequence to that previously reported for the wild-type allele (27). Thus, the loss of the revertant phenotype in these type-2 transformants was associated with disruption of the mutant allele and retention of the wild-type allele.

The sequence of *RIM101* was examined in a second independent revertant, CEM-2. PCR amplification using genomic DNA as the template produced a mixed product derived from both alleles. Direct sequencing of this product demonstrated that the chromatographic peak corresponding to position +1751 of the open reading frame consistently exhibited overlapping nucleotides of C and A. Sequence analysis of multiple subclones of the PCR product demonstrated two types of clones, one having a C at position 1751, the other having an A. The cytosine corresponds to the wild-type sequence. The adenine transversion converts serine codon 584 to a nonsense codon. This would truncate the protein by 78 residues. Thus, two independent revertants, CEM-1 and CEM-2, had both acquired a nonsense mutation in one allele of *RIM101* and both mutations resulted in a carboxy-terminal truncation of the predicted protein.

The mutant alleles of *RIM101* confer the revertant phenotypes. To test whether the mutant alleles of *RIM101* were causative of the revertant phenotype, the mutant allele recovered from CEM-1, designated *RIM101-1426*, or the one from CEM-2, designated *RIM101-1751*, were transformed into the *phr2Δ* mutant CFM-4 and integrated at the *URA3* locus. The Uri$^+$ transformants were selected at pH 7.0, and 10 *RIM101-1426* and 10 *RIM101-1751* transformants were characterized. Representative transformants CEM-16-1, containing *RIM101-1426*, and CEM-16-3, containing *RIM101-1751*, are shown in Fig. 3. Like the revertants CEM-1 and CEM-2, the transformants acquired the ability to grow at pH 4.0 (Fig. 3b). Similarly, the transformants had gained the ability to filament at pH 4.0, and the frequency of germ tube formation was similar to that in the revertants, 89% ± 4% (Fig. 3c). Northern blot analysis demonstrated that, as in the revertants, expression of *PHR1* was pH independent (Fig. 3d). Identical phenotypes were observed in all 10 transformants, and no differences were observed between cells receiving *RIM101-1426* or *RIM101-1751*. In contrast, introduction of the wild-type allele recovered from revertant CEM-1 or CEM-2 had no obvious phenotypic consequences in the 10 transformants examined. Representative transformants CEM-31-1 and CEM-31-3 are shown in Fig. 3. Thus, a single copy of the mutant allele was sufficient to impart the pleiotropic phenotypes observed in the revertants. Also, since these strains contained, in addition to the mutant allele, both wild-type alleles at the *RIM101* locus, *RIM101-1426* and *RIM101-1751* were at least partially dominant to the wild type.

The phenotype conferred by the mutant alleles was independent of the status of the *PHR2* locus. When *RIM101-1426* was transformed into CAF3-1, which is wild type for *PHR2*, filamentation and *PHR1* expression became pH independent (data not shown). Since this transformant, strain CAF3-1-16, was wild type for *PHR2*, this allowed examination of the effect of the mutation on *PHR2* expression. In wild-type cells *PHR2* is repressed under alkaline growth conditions and expressed at acidic pH. In *rim101Δ* mutants, *PHR2* is constitutively expressed (6, 27). In the presence of *RIM101-1426*, expression of *PHR2* was not detected at either acidic or alkaline pH (Fig. 3e). As a control, the wild-type allele of *RIM101* allele was introduced into CAF3-1, producing strain CAF3-1-31. This

a.)

CFM-2 (*phr2Δ*)

↓

CFM-4 (*phr2Δ, ura3Δ*)

↓

CEM-1 (*phr2Δ, ura3Δ, RIM101/RIM101-1426*)

↙ ↘

CEM-5A and CEM-5B   CEM-6A and CEM-6B
(*phr2Δ, rim101/RIM101-1426*)   (*phr2Δ, RIM101/rim101-1426*)

b.)

pH 4 | pH 7

c.)

PHR1

ACT1

pH 4   pH 8

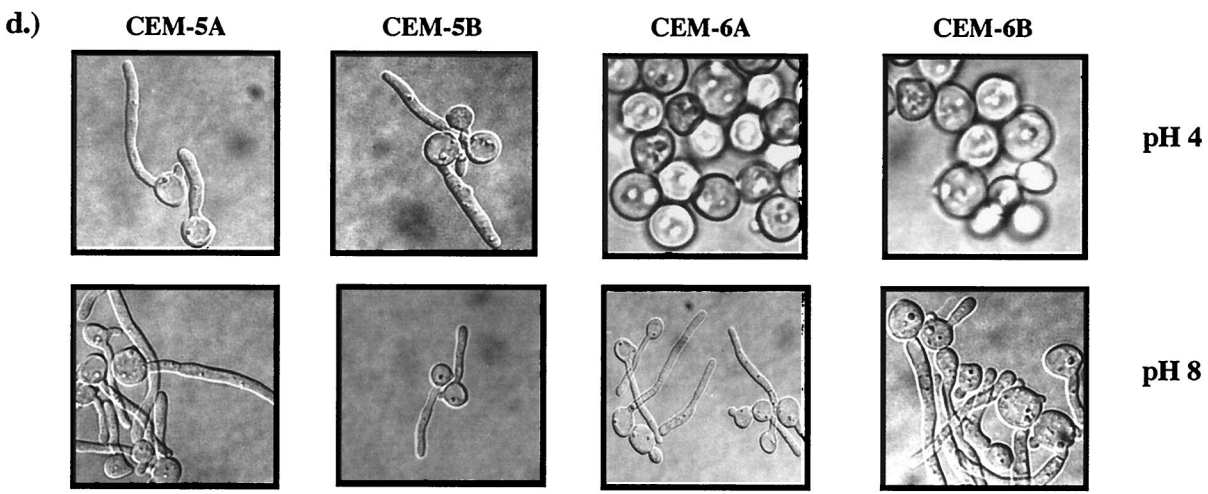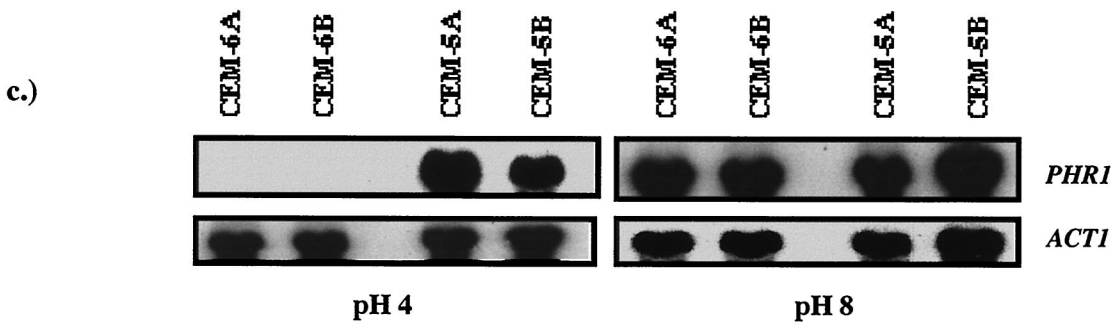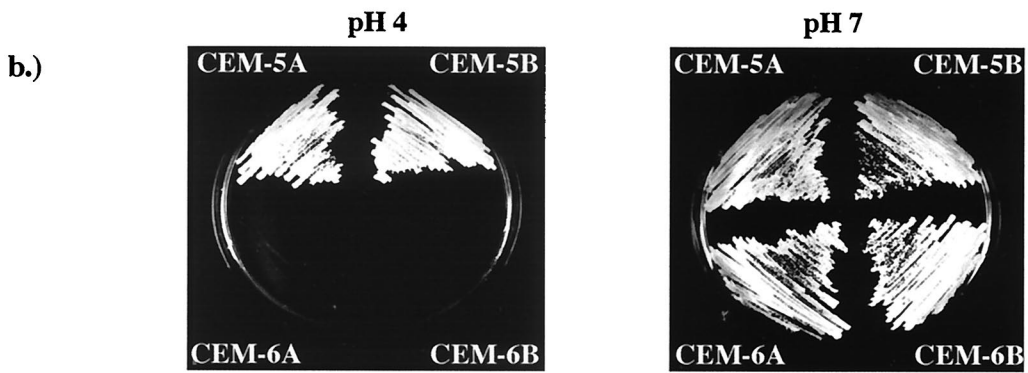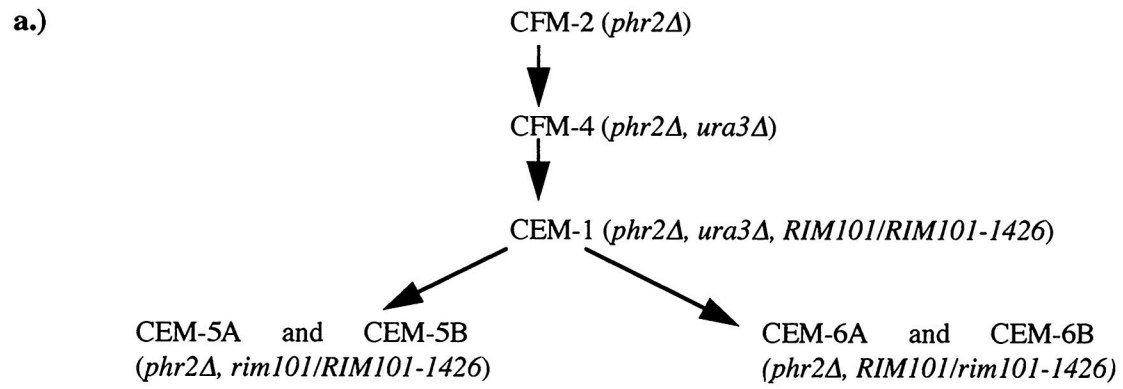**d.)**

CEM-5A   CEM-5B   CEM-6A   CEM-6B

pH 4

pH 8

FIG. 2. Effect of *RIM101* disruption in CEM-1. (a) Genealogy of revertant CEM-1 and transformants CEM-5A, CEM-5B, CEM-6A, and CEM-6B. The relevant genotypes are indicated. (b) Growth of strains on YNB at pH 4.0 or 7.0 following 48 h at 30°C. (c) *PHR1* expression as a function of ambient pH. RNA was isolated from the indicated strains cultured in medium 199 at the pH indicated below the panels. The upper panel shows the Northern blot hybridized with *PHR1*. The lower panel was hybridized with *ACT1*. (d) Morphology of the indicated strains 3 h postinoculation into medium 199 at pH 4.0 or pH 8 and 37°C.
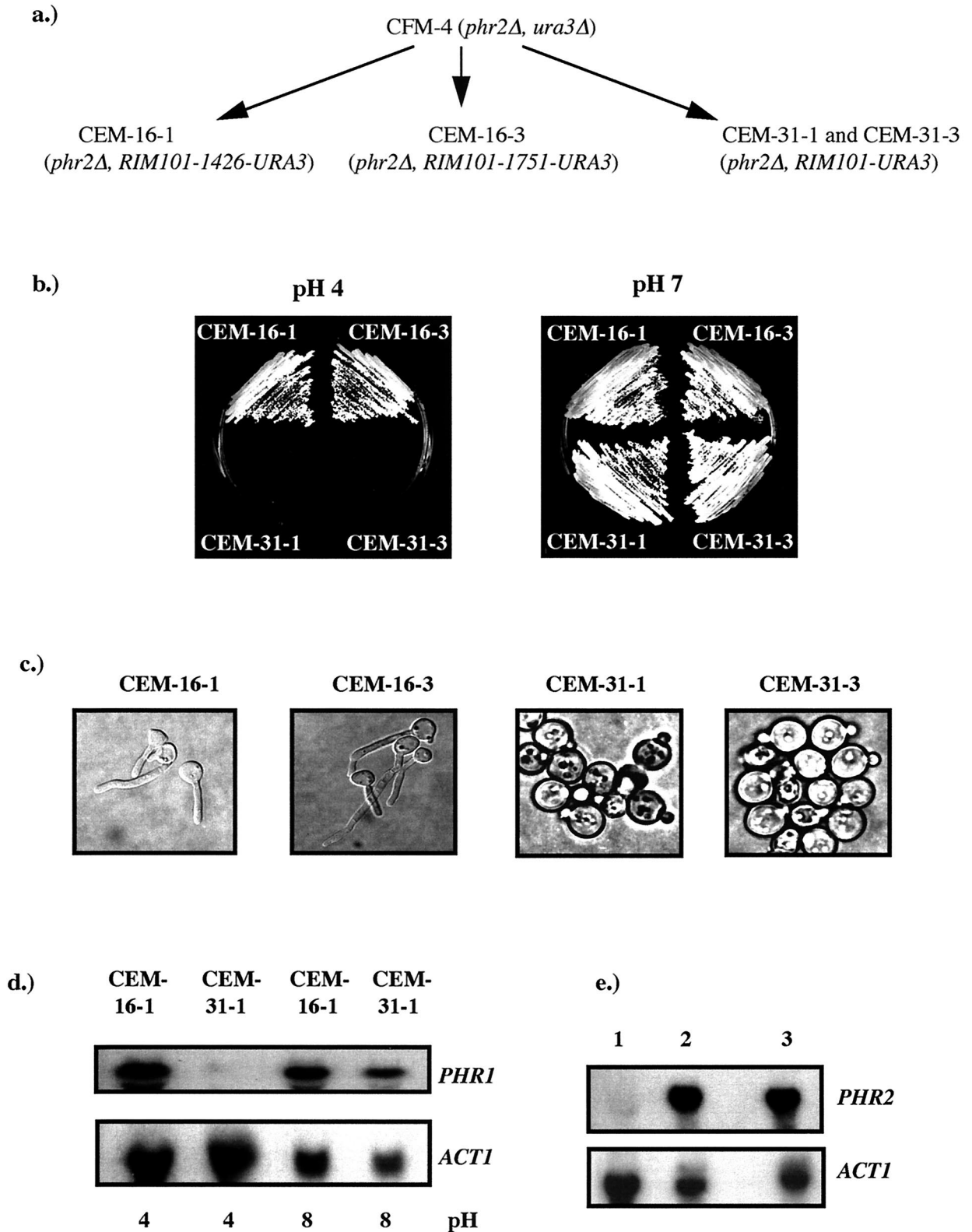
FIG. 3. Effect of mutant alleles of *RIM101*. (a) Genealogy of strains. Transformation of the mutant alleles *RIM101-1426* and *RIM101-1751* into CFM-4 produced strains CEM-16-1 and CEM-16-3, respectively. Introduction of the wild-type allele produced control strains CEM-31-1 and CEM-31-3. (b) Growth of strains on YNB at pH 4.0 or 7.0 after 48 h at 30°C. (c) Morphology of the indicated strains 3 h postinoculation into medium 199 at pH 4.0 and 37°C. (d) *PHR1* expression as a function of ambient pH. RNA was isolated from the indicated strains cultured in medium 199 at the pH indicated below the panels. The upper panel shows the Northern blot hybridized with *PHR1*. The lower panel was hybridized with *ACT1*. (e) Effect of *RIM101-1426* on *PHR2* expression. RNA was isolated from CAF3-1-16 (lane 1), CAF3-1-31 (lane 2), and SC5314 (lane 3) grown in medium 199 at pH 4. The Northern blots were hybridized with *PHR2* or *ACT1*, as indicated. CAF3-16-1 contains *RIM101-1426*. CAF3-31-1 contains the wild-type allele. SC5314 is a wild-type control.

had no effect on filamentation or expression of *PHR2* (Fig. 3e and data not shown).

Multiple copies of *RIM101* partially suppress the temperature requirement for filamentation. Although the mutant alleles bypassed the pH requirement for filamentation, the strains were not constitutively hyphal and still required a temperature of approximately 37°C for filamentation to occur. Fortuitously, we observed that the mutant allele could act as a multicopy suppressor of the temperature requirement. The revertant CEM-1 was transformed with plasmid pEM-16-1 containing the mutant allele *RIM101-1426*. Southern blot analysis of the transformants demonstrated that some had integrated a single copy of the plasmid at the *URA3* locus and others had integrated multiple tandem copies. Integration of a single copy was indicated by a 1-kb band in addition to the 8.2-kb *Pst*I hybridization band present in the parental strain CEM-1 (Fig. 4a). The intensity of the 1-kb band was enhanced in some of the transformants (Fig. 4a), in comparison to control hybridizations with the *ACT1* probe, indicating integration of multiple tandem copies. Two transformants containing a single copy of the plasmid, represented by strain CEM-7, and four containing multiple tandem copies, represented by strain CEM-8, were examined. All of these transformants maintained the ability to filament at pH 4.0 or pH 7.5 at 37°C (Fig. 4b). As with wild-type strains, no filamentation occurred at 25°C regardless of the pH (data not shown). However, at 29°C, germ tube formation was >70% for those transformants containing multiple copies of the plasmid but <1% for transformants with a single integrated copy. Wild-type cells and the parental strain CEM-1 also failed to filament at this temperature. Low-temperature filamentation was independent of pH and was observed at both pH 4.0 and pH 7.5. The multicopy integrants also exhibited extensive filamentation on agar-solidified medium at 29°C (data not shown). Low-temperature filamentation was also observed when multiple copies of *RIM101-1426* were introduced into CAF3-1, indicating that the phenotype was independent of the presence or absence of *PHR2* (data not shown).

The requirement for multiple copies of *RIM101-1426* was not due to incomplete dominance of the mutant allele. This possibility was raised by the fact that the CEM-1 transformants contain one wild-type allele of *RIM101* and CAF3-1 transformants contain two. The wild-type allele could conceivably interfere with complete expression of the mutant allele. However, germ tube formation by strain CEM-5A was restricted to 37°C. CEM-5A was constructed by disruption of the wild-type allele of *RIM101* in the revertant CEM-1 and thus contains a single mutant allele, *RIM101-1426*. Similarly, germ tube formation by strain CEM-10 was also restricted to 37°C. CEM-10 was derived from strain CEM-5A by integration of a single copy of plasmid pEM-16-1. Thus, CEM-10 lacked the wild-type allele and contained two copies of *RIM101-1426*. Analysis of 11 additional strains analogous to CEM-10 gave the same result. These data indicated that more than two copies of *RIM101-1426* are required to suppress the temperature requirement for filamentation.

As a control, strains containing multiple copies of the wild-type allele were constructed by transformation of CAF3-1 (*PHR2/PHR2*) and CFM-4 (*phr2Δ/phr2Δ*) with plasmid pEM-31-1. Unexpectedly, in both backgrounds, strains containing multiple tandem copies of the plasmid exhibited a high frequency of germ tube formation at 29°C, comparable to strains with multiple copies of the mutant allele. Unlike the transformants harboring *RIM101-1426*, however, filamentation was restricted to neutral pH (data not shown). To ensure that suppression was not due to multiple copies of the vector sequences or the marker gene, strains CEM-1 and CAF3-1 were transformed with plasmid pSM-2, which lacks the *RIM101* sequences present in pEM-16-1 and pEM-31-1. Germ tube formation by strains containing multiple copies of pSM-2 was restricted to 37°C. Thus, multiple copies of either the wild-type or mutant allele of *RIM101* can partially suppress the temperature requirement for filamentation.

***RIM101-1426* does not bypass *EFG1*.** *EFG1* encodes a transcription factor that lies downstream of a cAMP-dependent protein kinase signal pathway (21, 35, 37). Deletion of *EFG1* prevents germ tube formation and restricts morphological development largely to pseudohyphae under a wide variety of inducing conditions (21, 35). The developmental defect imparted by an *efg1Δ* mutation is potentiated by deletion of *CPH1*, which encodes a transcription factor at the terminus of a mitogen-activated protein kinase cascade (20, 21). An *efg1Δ cph1Δ* mutant is restricted entirely to the yeast morphology under most, but not all, conditions (21, 28, 34), even though the *cph1Δ* mutation alone does not prevent germ tube formation in liquid medium and affects filamentation on only a few solid induction media (20). The dominant active alleles of *RIM101* provided the opportunity to access the interaction of the pH response pathway with these other developmental pathways.

Strains JKC18 (*cph1Δ*), HLC67 (*efg1Δ*), and CDB1 (*efg1Δ cph1Δ*) were transformed with plasmid pEM-16-1 containing *RIM101-1426*, plasmid pEM-31-1 containing the wild-type allele, or pSM-2, the base vector. Single-copy integration events were verified by Southern analysis, and five such isolates from each transformation were assessed for their ability to form germ tubes and to form hyphae on solid medium at pH 4.0 and 37°C. As shown in Fig. 5b and c, germ tube formation and filamentation in liquid medium and on agar were both blocked by the *efg1Δ* mutation either alone or in combination with the *cph1Δ* mutation. The *cph1Δ* mutation alone did not interfere with the ability of *RIM101-1426* to promote filamentation at acidic pH (Fig. 5b and c). Control strains transformed with the wild-type allele or vector alone were identical to the parental mutants. These results demonstrate that the filamentation phenotype conferred by *RIM101-1426* is *EFG1* dependent.

The inability of *RIM101-1426* to bypass the *efg1Δ* mutation was also evident in its lack of effect on developmental gene expression. *HWP1* encodes a cell wall protein whose expression is induced in filamentous cells and requires *EFG1* for expression (32, 36). *HWP1* was not expressed in the *efg1Δ* mutant in the presence or absence of *RIM101-1426* (Fig. 6).

pH-dependent gene expression does not require *EFG1*. Since *EFG1* was required for expression of the filamentation phenotype, the effect of *EFG1* on the gene expression phenotype of the *RIM101-1426* mutation was examined. The presence of the activated allele of *RIM101* in HLC67-16 (*efg1Δ RIM101-1426*) resulted in expression of *PHR1* at pH 4.0, as was observed in an *EFG1* wild-type background (Fig. 3 and 6). As previously observed (29), *PHR1* was not detected in the wild-type strain SC5314 at pH 4.0 (data not shown). Thus, the interaction between *RIM101* and *EFG1* that is required for filamentation is not required for pH-dependent gene regulation.

DISCUSSION

In examining spontaneous extragenic suppressors of the pH-conditional growth defect of *phr2Δ* mutants, we identified mutations in the *C. albicans* pH response regulator *RIM101* that bypassed the pH requirement for hyphal development. These results suggest that *RIM101* is the key element controlling the
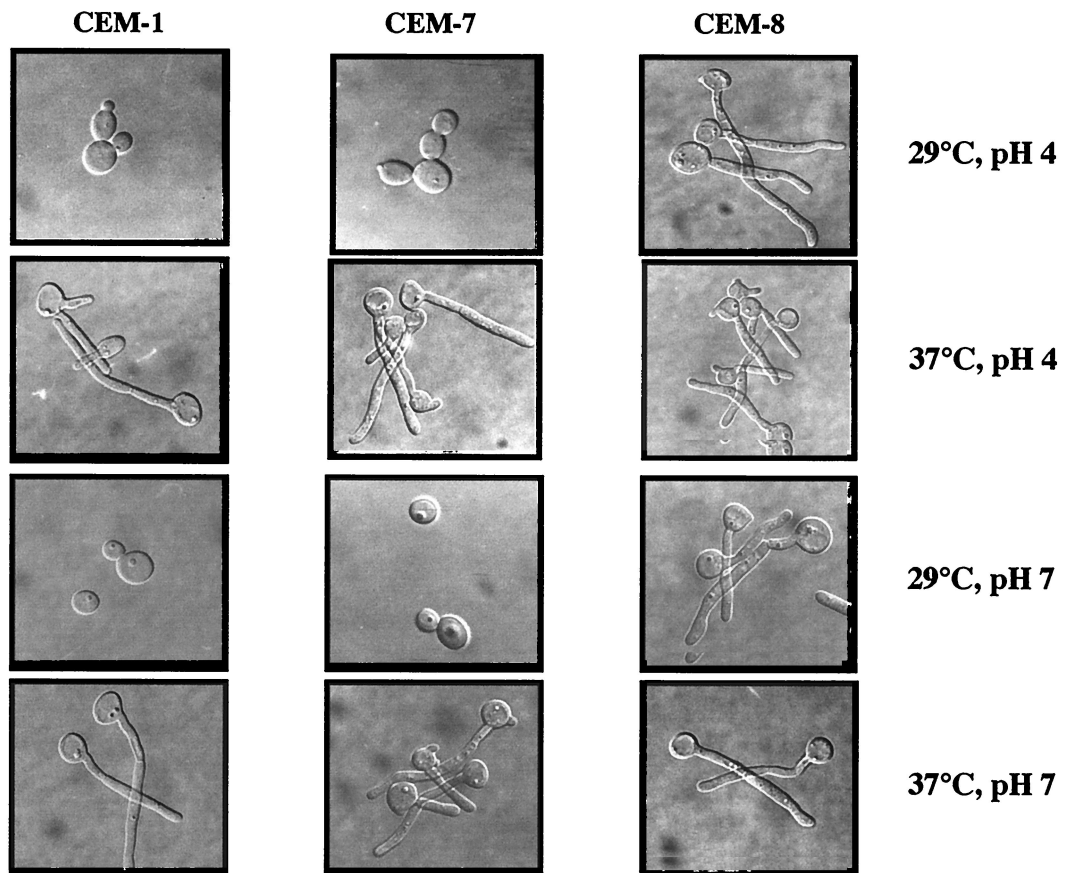
a)
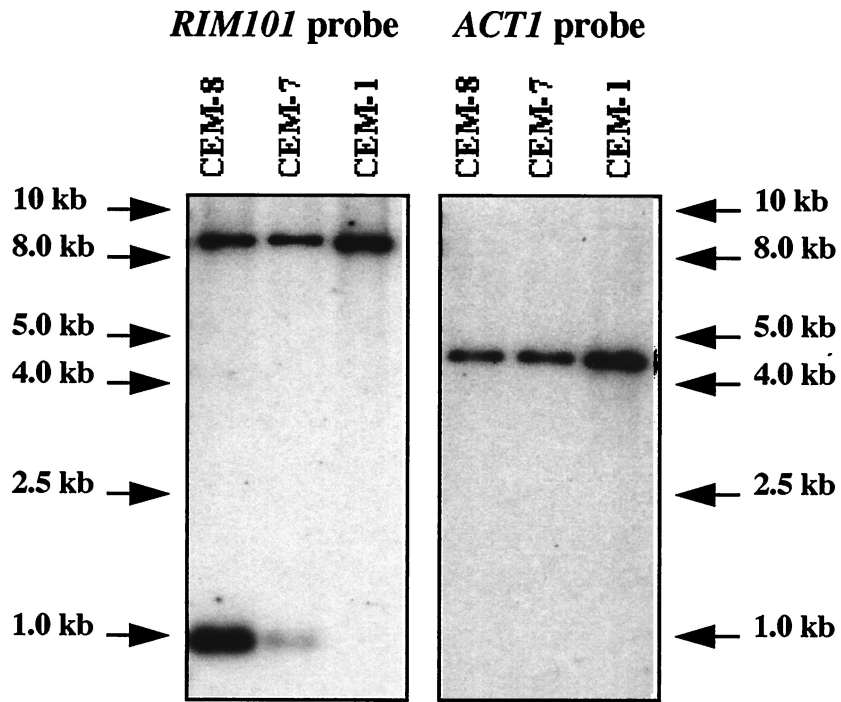
b.)

FIG. 4. Multicopy suppression of temperature restriction. (a) Equal amounts of genomic DNA from strain CEM-8 [*RIM101/(RIM101-1426)$_n$≥3*], CEM-7 (*RIM101/RIM101-1426/RIM101-1426*), and the parental strain CEM-1 (*RIM101/RIM101-1426*) were digested with either *Pst*I (left panel) or *Eco*RI (right panel). The Southern blots were hybridized with *RIM101* or *ACT1*, as indicated. (b) Morphology of CEM-1, CEM-7, and CEM-8 following 3 h of incubation in medium 199 at pH 4.0 or pH 7.0 and 29 or 37°C.
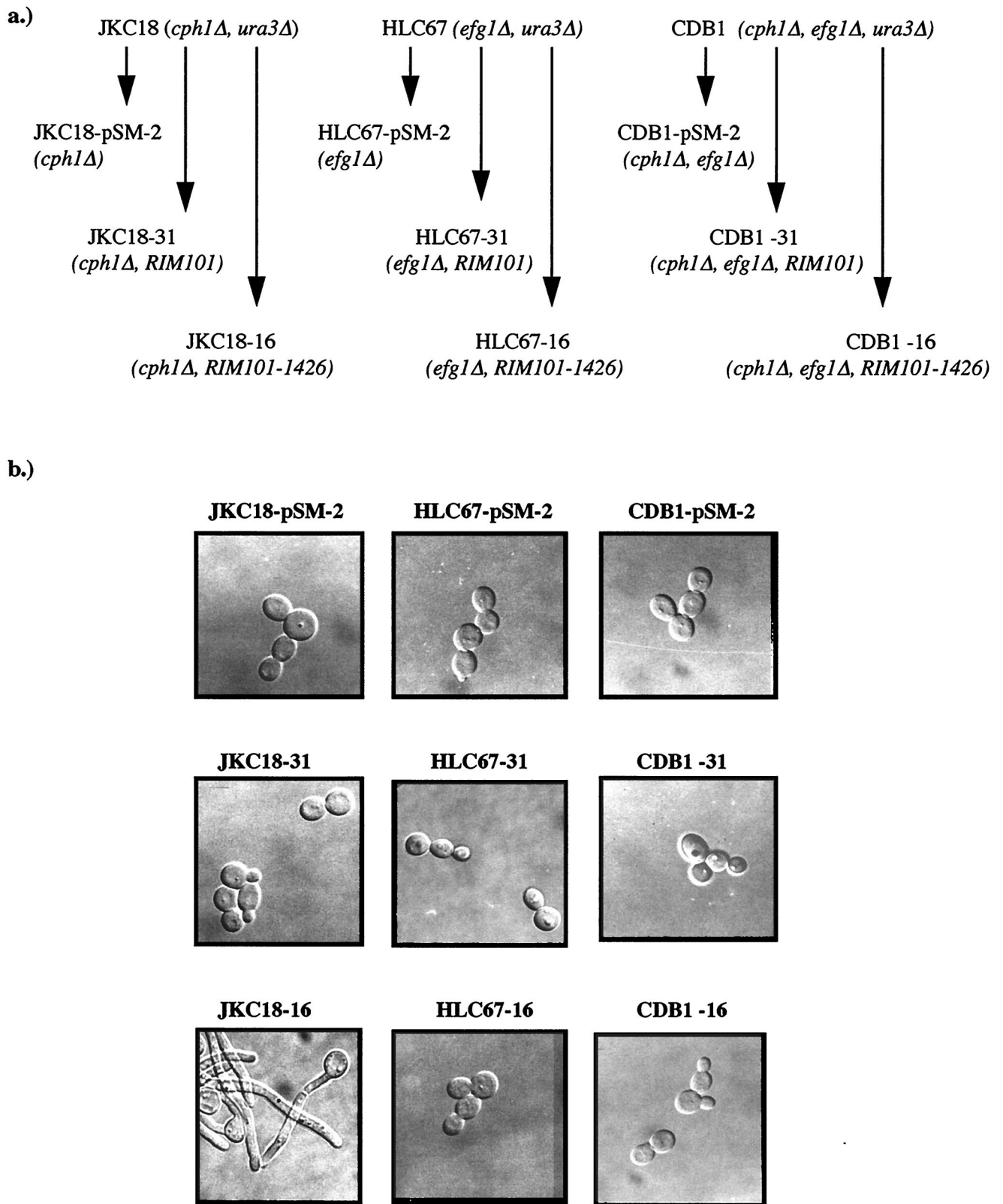
a.)

JKC18 *(cph1Δ, ura3Δ)*

JKC18-pSM-2
(cph1Δ)

JKC18-31
(cph1Δ, RIM101)

JKC18-16
(cph1Δ, RIM101-1426)

HLC67 *(efg1Δ, ura3Δ)*

HLC67-pSM-2
(efg1Δ)

HLC67-31
(efg1Δ, RIM101)

HLC67-16
(efg1Δ, RIM101-1426)

CDB1 *(cph1Δ, efg1Δ, ura3Δ)*

CDB1-pSM-2
(cph1Δ, efg1Δ)

CDB1 -31
(cph1Δ, efg1Δ, RIM101)

CDB1 -16
(cph1Δ, efg1Δ, RIM101-1426)

b.)

FIG. 5. Effect of mutations in *CPH1* and *EFG1* on expression of *RIM101-1426*. (a) Genealogy of strains. (b) Morphology of strains 3 h postinoculation into medium 199 at p/H 4.0 and 37°C. (c) Colony morphology following 6 days of incubation on medium 199 at pH 4.0 and 37°C.

c.)
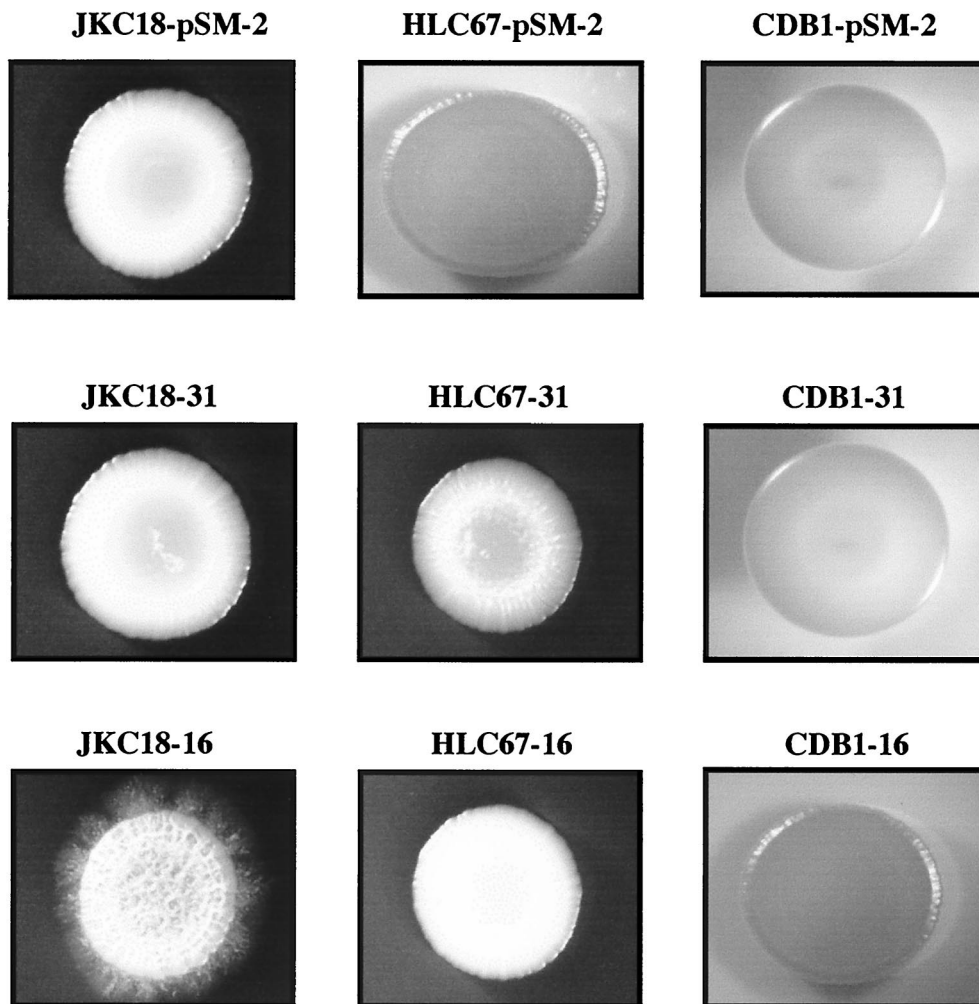
FIG. 5—*Continued.*

pH dependence of dimorphism in vitro. The mutations resulted in a gain of function at acidic pH, as evidence by the expression of *PHR1* at pH 4.0. Normally *PHR1* is not expressed below pH 5.5, and its expression is dependent upon *PRR1/RIM101* (26, 27). The aberrant expression of *PHR1* in the revertants is likely to account for the restoration of growth, since forced expression of *PHR1* was previously shown to complement the loss of *PHR2* (23).

The dominant gain of function associated with these mutations is clearly consistent with the current model of pH-dependent gene expression developed in *A. nidulans* and with related data from studies of other fungi (17, 19, 39). Both mutant alleles contained a nonsense mutation resulting in premature termination of the open reading frame. In *RIM101-1426*, a C-to-T transition at nucleotide 1426 introduced an ochre codon at position 476 of the coding region, truncating the 661-residue native protein by 186 amino acids. Similarly, a C-to-A transversion at position 1751 of *RIM101-1751* converted the codon for Ser-584 to an ochre stop codon, truncating the protein by 78 residues. Rim101p is homologous to the zinc finger-containing transcription factor PacC of *A. nidulans*,

Rim101p of *S. cerevisiae*, and the *Y. lipolytica* homolog YlRim101p (17, 19, 27, 39). PacC is synthesized as a 687-residue inactive precursor, which is activated when cells are cultured at an alkaline pH. Activation occurs by proteolysis
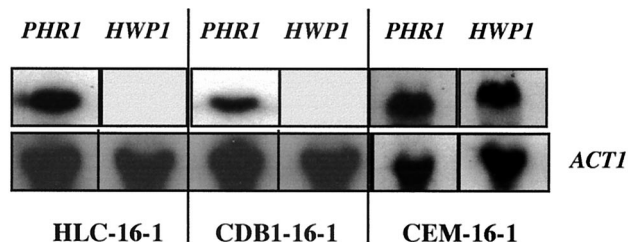

FIG. 6. Effect of *EFG1* on expression of *RIM101-1426*. RNA was isolated from HLC67-16-1 (*efg1Δ CPH1 RIM101-1426*), CDB1-16-1 (*efg1Δ cph1Δ RIM101-1426*), and CEM-16-1 (*EFG1 CPH1 RIM101-1426*) 3 h postinoculation into medium 199 at pH 4 and 37°C. The Northern blot was hybridized with *PHR1*, *HWP1*, or *ACT1*, as indicated.

around residues 252 to 254, approximately 90 amino acids carboxy-terminal to the zinc finger domain (9, 25). The carboxy terminus is essential to the pH dependence of activation and appears to control accessibility of the proteolytic site (9, 25). Thus, mutations that cause premature termination and loss of the carboxy terminus result in proteolytic activation irrespective of ambient pH (9, 25). Since PacC is required for transcriptional activation of alkaline-expressed genes and repression of acid-expressed genes, these truncating mutations can result in constitutive expression of alkaline-expressed genes and constitutive repression of acid-expressed genes (39). Similar effects are observed for truncated alleles of yeast *RIM101* and *YlRIM101* (17, 19). The observation that *RIM101-1426* and *RIM101-1751* allow expression of the alkaline-induced gene *PHR1* at acidic pH and cause constitutive repression of *PHR2* is entirely analogous to PacC.

The ability of *RIM101-1426* and *RIM101-1751* to relieve the pH dependence of hyphal development suggests that activation of Rim101p is the limiting factor for filamentation at acidic ambient pH. This result complements previous studies demonstrating that *RIM101* is required for filamentation (27). However, the phenotype of the *RIM101-1426* and *RIM101-1751* mutations is in contrast to a previous report in which site-specific mutagenesis was used to introduce a premature stop codon in *RIM101* (6). This mutation, located at codon 405 of the reported sequence (6), corresponding to codon 462 of the putative full-length open reading frame (27), was shown to suppress the loss of upstream components of the pH response pathway but did not overcome the inhibition of filamentation at acidic pH (6). These phenotypic differences may reflect intrinsic differences in the mutant alleles that affect, for instance, the proteolytic processing or stability of the truncated protein. Allele-specific variations in phenotype have been observed for PacC mutations (39).

An unexpected observation was that *RIM101* could act as a multicopy suppressor of the temperature requirement for filamentation. In typical pH-regulated dimorphism, a shift from acid to neutral induces filamentation only if the ambient temperature is near 37°C. Work by Soll and colleagues (4) showed that lowering the temperature below a critical threshold of 34°C prevents filamentation irrespective of the ambient pH. The presence of four or more copies of the wild-type *RIM101* permitted extensive filamentation to occur at temperatures below 30°C. Despite the diminished temperature restriction, morphological development was still pH dependent. If, however, multiple copies of the activated alleles were introduced, both the temperature and pH restrictions were removed. Suppression of the temperature requirement may be a direct effect if the stability of Rim101p or its proteolytic activation is normally limiting at temperatures below 34°C. Alternatively, elevated Rim101p may bypass temperature-dependent limitations on expression of a downstream function. In either case, this observation suggests that two environmental signals, pH and temperature, converge on common molecular targets. Integration of these signals may be important in establishing infection of external versus internal niches or survival outside the host or may facilitate transmission between hosts.

Despite the prominent role of *RIM101* in controlling hyphal development, it does not act independently. An *efg1Δ* mutation was epistatic to *RIM101-1426* and prevented filamentation. This result does not distinguish whether *RIM101* and *EFG1* act in parallel or within a single pathway. If *RIM101* and *EFG1* function within the same regulatory pathway, then this result suggests that *EFG1* lies downstream of *RIM101*. The observation that the *efg1* mutation did not prevent *RIM101-1426* activation of *PHR1* expression demonstrated that *EFG1* is not interposed within the pathway controlling pH-dependent gene expression. However, this does not rule out the possibility of their functioning within the same pathway to control filamentation. In this regard it might be noted that *EFG1* lies downstream of *TPK2*, which encodes a cAMP-dependent protein kinase (35). Rim101p of *S. cerevisiae* contains a functionally significant recognition site for cAMP-dependent protein kinases (38). Although this site is not conserved in the *C. albicans* homolog, two potential phosphorylation sites are present, and this could provide a regulatory connection between *TPK2*, *RIM101*, and *EFG1*.

Although *RIM101* appears to impose a pH dependence on filamentation in vivo, its role in controlling morphological development during infection remains unclear, since extensive filamentation occurs during infection of acidic host niches (7). This suggests that in the acid niche, the requirement for *RIM101* is bypassed or an alternative signal promotes Rim101p activation.

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