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RESEARCH ARTICLE

**Thomas D. Edlind · Karl W. Henry
John-Paul Vermitsky · Merritt P. Edlind
Shriya Raj · Santosh K. Katiyar**

Promoter-dependent disruption of genes: simple, rapid, and specific PCR-based method with application to three different yeast

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Abstract PCR product-based gene disruption has greatly accelerated molecular analysis of *Saccharomyces cerevisiae*. This approach involves amplification of a marker gene (e.g., *URA3*) including its flanking regulatory (promoter and polyadenylation) regions using primers that include at their 5′ ends about 50 bases of homology to the targeted gene. Unfortunately, this approach has proved less useful in organisms with higher rates of non-homologous recombination; e.g., in the yeast *Candida glabrata*, desired recombinants represent ≤ 2% of transformants. We modified the PCR-based approach by eliminating marker-flanking regions and precisely targeting recombination such that marker expression depends on the regulatory sequences of the disrupted gene. Application of this promoter-dependent disruption of genes (PRODIGE) method to three *C. glabrata* genes (*SLT2*, *LEM3*, and *PDR1*) yielded desired recombinants at frequencies of 20, 31, and 11%, the latter representing a weakly expressed gene. For *Candida albicans LEM3* and *RHO1*, specificity was 79–95% for one or both alleles, > sixfold higher than the published results with conventional PCR-based gene disruption. All 5 *C. glabrata* and *C. albicans* mutants had predicted phenotypes of calcofluor hypersensitivity (*slt2*Δ and *RHO1*/*rho1*Δ), cycloheximide hypersensitivity (*pdr1*Δ), or miltefosine resistance (*lem3*Δ and *lem3*Δ/*lem3*Δ). PRODIGE application to the *S. cerevisiae PDR5* gene in strains with and without the Pdr1–Pdr3 transcriptional activators of this gene confirmed that transformant yield and growth rate depend on promoter strength. Using this *PDR5* promoter-*URA3* recombinant, we further demonstrate a simple extension of the method that yields regulatory mutants via 5-fluoroorotic acid selection. PRODIGE warrants testing in other yeast, molds, and beyond.

Keywords *Candida glabrata* · *Candida albicans* · *Saccharomyces cerevisiae* · Gene deletion · Transformation

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T. D. Edlind (✉) · K. W. Henry · J.-P. Vermitsky
M. P. Edlind · S. Raj · S. K. Katiyar
Department of Microbiology and Immunology,
Drexel University College of Medicine, 2900 Queen Lane,
Philadelphia, PA, 19129, USA
E-mail: tedlind@drexelmed.edu
Tel.: +1-215-9918377
Fax: +1-215-8482271

Introduction

Targeted gene disruption represents a cornerstone of molecular genetics, with application to a wide range of organisms. Gene disruption conventionally employs a cassette containing two regions of homology to the targeted gene flanking a selectable marker gene (nutritional or drug resistance) that includes promoter and polyadenylation regions required for marker expression. Multiple DNA cloning steps are generally required to generate these cassettes, incorporating flanking homology regions hundreds or thousands of bp long.

An important modification, now widely applied to the model yeast *Saccharomyces cerevisiae*, involves the use of PCR rather than cloning to generate disruption cassettes (McElver and Weber 1992; Baudin et al. 1993; Lorenz et al. 1995). The PCR primers typically include about 20 bases at their 3′ ends for amplification of the marker gene including promoter and polyadenylation regions, along with 30–60 bases at their 5′ ends that are homologous to the targeted gene. In *S. cerevisiae*, these short homology regions are adequate to achieve specificities of > 50% homologous recombinants versus total transformants (Manivasakam et al. 1995).

Unfortunately, in other organisms, PCR product-based gene disruption has proven much less useful, and this has been generally attributed to the need for longer regions of homology. In the diploid yeast *Candida albicans*, specificities of 4–21% (average = 13%) were achieved using 60 bp homology regions for alleles of

three different genes (Wilson et al. 1999, 2000). However, another laboratory reported that 100 bp of homology were required for efficient gene disruption in *C. albicans*, with 75 bp yielding $<1\%$ specificity (Gola et al. 2003). In the haploid *Candida glabrata*, homology regions of 50 bp yielded specificities of $\leq 2\%$ (Cormack and Falkow 1999; Weig et al. 2001). Attempts have been made to address these limitations by using overlap or fusion PCR protocols designed to generate longer homology regions (Wach 1996; Willins et al. 2002); however, these methods require multiple PCR reactions and considerable optimization to generate the necessary yield and purity of DNA.

With any of the above methods, specificity is reduced because expression of the selection marker is largely independent of its integration site. We describe here a simple modification of conventional PCR product-based gene disruption: cassettes are generated with a promoter-less marker gene such that its expression is dependent, following recombination, on the promoter of the targeted gene. Promoter-dependent disruption of genes (PRODIGE) dramatically enhanced specificity in *C. glabrata* and *C. albicans*, and is likely to be applicable to other organisms as well. Studies in *S. cerevisiae* with the *URA3* marker suggest that only minimal levels of target gene expression are required; furthermore, PRODIGE can be readily extended to a selection system for regulatory mutants.

Materials and methods

Strains, plasmids, and media

Candida glabrata strains BG14 (*ura3::Tn903NeoR*; Cormack and Falkow 1999) and 200989 (*ura3 his3Δtrp1Δ*;Kitada et al. 1995) were obtained from B. Cormack (Johns Hopkins University) and the ATCC (Manassas, VA), respectively. *C. glabrata* 8512-11 (*ura3*) was isolated in our lab as a spontaneous 5-fluoroorotic acid (5FOA)-resistant derivative of azole resistant clinical isolate 8512 (Vermitsky and Edlind 2004). *C. albicans* strain BWP17 (*ura3Δ/ura3Δ, his1Δ/his1Δ, arg4Δ/arg4Δ*; Wilson et al. 2000) was obtained from A. Mitchell (Columbia University). *S. cerevisiae* nA1Δ 3Δ (*MATa ura3-52 his3-Δ200 leu2-Δ1 trp1-Δ63 pdr1::TRP1 pdr3::HIS3*) (Delaveau et al. 1994) was obtained from K. Kuchler (University and Biocenter of Vienna) and IPY36 (*MATa his3Δ200 leu2Δ1 ura3-52 trp1ΔhisG*) was from S. Berger (Wistar Institute). Plasmids YEp24 and pRS414 (Christianson et al. 1992) were obtained from J. Nickels (Drexel University), pDDB57 (Wilson et al. 1999) was from A. Mitchell, and pFA-ARG4 (Gola et al. 2003) was from J. Wendland (Friedrich-Schiller University). Routine cultures employed YPD medium (1% yeast extract, 2% peptone, 2% dextrose); this was supplemented with 80 μg/ml uridine for *C. albicans* BWP17 and its derivatives. Transformants were selected on synthetic defined medium with dextrose and appropriate supplements (DOB plus CSM-URA, CSM-TRP, or CSM-ARG; Qbiogene/BIO 101) including uridine where indicated.

Primers

All primer sequences are presented in Table 1. Primers for amplifying disruption cassettes were 80 nucleotides in length and PAGE purified by the manufacturer (Integrated DNA Technologies), except for *C. glabrata ADE2* disruptions, which employed 60 nucleotide unpurified primers. The PRODIGE primers were designed following the strategy shown in Fig. 1a, with their 3′ 20–27 nucleotides (Tm ≈ 60°C) derived from the termini of the marker gene coding sequence (e.g., *URA3*) and the remainder derived from the flanking sequences of the targeted gene. Marker gene coding sequences were obtained from YEp24 or pRS414 (*S. cerevisiae URA3* and *TRP1*; GenBank accession number L09156 and U03448, respectively), pDDB57 (*C. albicans URA3*; AF173953), and pRS-ARGΔSpe (*C. albicans ARG4*; AF173956). *S. cerevisiae* gene and protein sequences were obtained from the *Saccharomyces* Genome Database (http://www.yeastgenome.org), *C. glabrata* sequences from the Genolevures database (http://cbi.labri.fr/Genolevures/elt/CAGL), and *C. albicans* sequences from the Stanford database (http://sequence-www.stanford.edu/group/candida) or from CandidaDB (http://genolist.pasteur.fr/CandidaDB). Primers for PCR confirmation (Fig. 1b; Table 1) were generally derived from upstream (u) regions of targeted genes along with internal (i) regions of the target and marker genes; the *C. albicans rho1*Δ confirmation primer derived from the downstream (d) region of the target gene.

PCR of disruption cassettes

Marker coding sequences were amplified with PRODIGE primers using the indicated plasmid DNA (purified by QIAprep spin, Qiagen) as template. Reactions (50 μl) included 0.2 mM dNTPs, 0.5 μM each primer, 1.5 units Ex-Taq (TaKaRa), 1 X Ex-Taq buffer, and about 20 ng of plasmid DNA. Amplification involved initial denaturation at 94°C for 2 min followed by 25–30 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 1 min, and extension at 70°C for 1.5 min; the final extension at 70°C was 4–5 min. Four separate reactions were run to increase yield and decrease the effects of mutations. The reactions were pooled, a sample checked by gel electrophoresis, and the remainder purified using Wizard SV PCR Clean-up System (Promega) with elution in 20–30 μl water.

Transformation and screening

Both the lithium acetate transformation procedure (Ausubel et al. 1997) and the Frozen-EZ Yeast Trans-

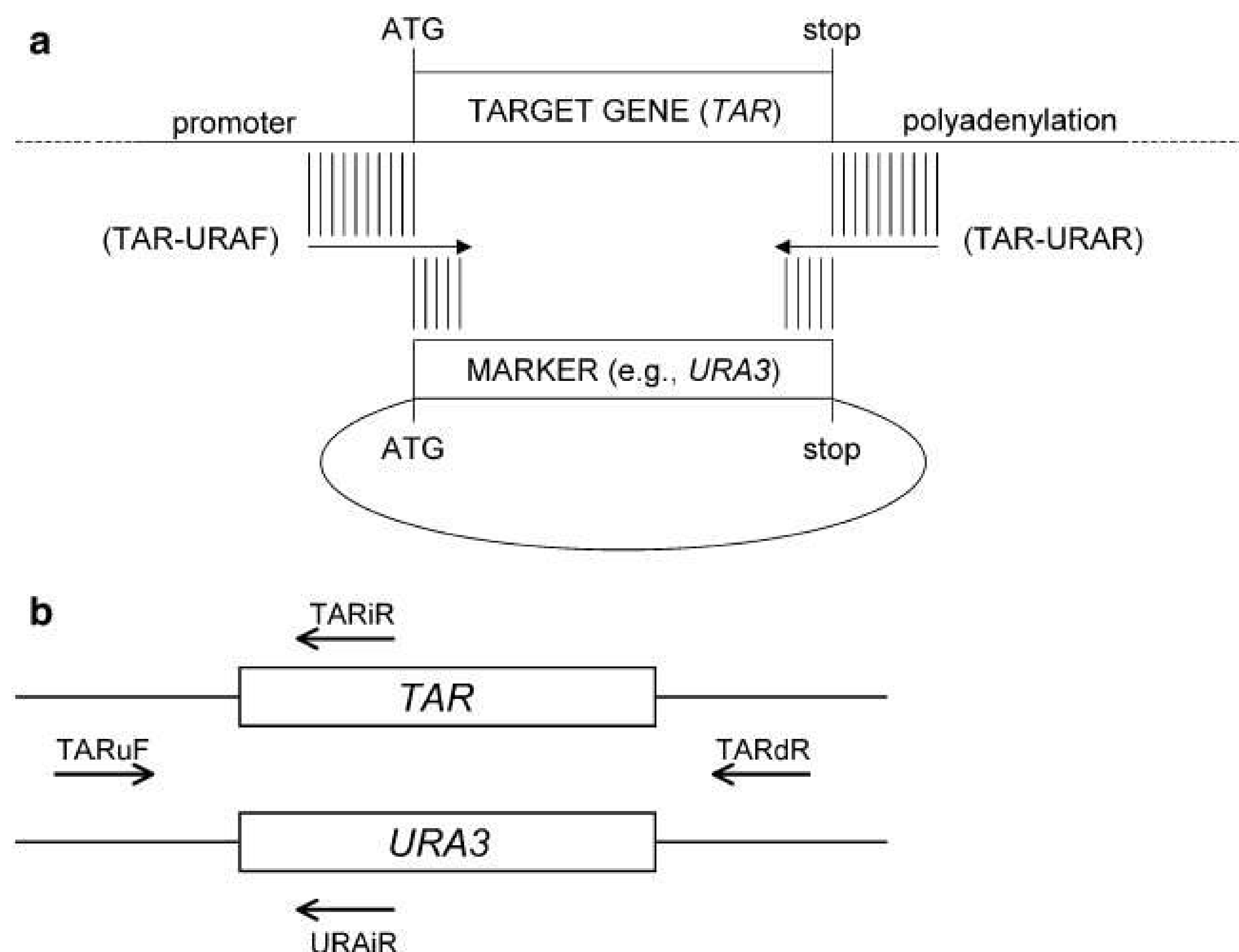

Fig. 1 **a** Diagram showing the design of PCR primers for the PRODIGE method, in which the target gene coding sequence (*TAR*) is precisely replaced with a marker coding sequence following homologous recombination. In this example, the marker is *S. cerevisiae URA3*, the PCR template is YEp24, and the forward and reverse primers are labeled TAR-URAF and TAR-URAR, respectively. **b** Diagram showing the design of primers for PCR confirmation of *TAR* disruption by *URA3*. TARuF, upstream forward primer; TARiR and URAiR, internal reverse primers; TARdR, downstream reverse primer. Primer lengths (*arrows*) are not to scale

formation II Kit (Zymo Research) were used, with the latter (using non-frozen cells) generating several-fold higher yields. In both cases, prepared cells were transformed with about 1 μg of PCR product (see above), and transformants were selected on appropriate medium with incubation at 30°C for 3–10 days. For PCR screening, genomic DNAs were prepared essentially as described (Ausubel et al. 1997). Briefly, isolated colonies from the transformation plate were cultured overnight in selective medium, pelleted, suspended in breaking buffer, glass beads, and buffer-saturated phenol and vortexed 2 min. Following centrifugation, the aqueous layer was reextracted with chloroform:isoamyl alcohol and the DNA ethanol precipitated. Following an ethanol wash and drying, the pellet was suspended in 100 μl water. The PCR reactions (25 μl) included 0.2 mM dNTPs, 1X reaction mix and 0.5 units Taq polymerase (Promega), 0.5 μM of indicated primers, and 1 μl of 1:10-diluted genomic DNA. Amplification conditions were as described above except that extension time was 1 min and cycle number was 25. Following this initial screen, selected transformants were streaked for isolation on YPD plates and the PCR screening of individual colonies was repeated.

Drug sensitivity assays

Drug sensitivities were examined by spotting about 500 cells from fresh cultures onto YPD plates with varying concentrations of calcofluor (Sigma-Aldrich), miltefosine (Cayman), or cycloheximide (Sigma) followed by incubation at 30°C for 2–3 days. For the *S. cerevisiae pdr5*Δ*::URA3* disruptant and its parent, growth rates of mid-log cultures were determined in DOB with CSM or CSM-URA broth at 30°C by measuring absorbance at 630 nm. Sensitivity to 5FOA (Zymo Research) was determined by broth microdilution assay (Vermitsky and Edlind 2004) in both YPD and synthetic defined medium at 30°C.

RNA blots and 5FOA-resistant mutants

The RNA was prepared, slot-blotted to nylon membranes, and hybridized as previously described (Vermitsky and Edlind 2004). Probes were amplified from *S. cerevisiae* genomic DNA using gene-specific primers and labeled with ^{32}P by random priming. To isolate 5FOA-resistant mutants, 4×10^7 cells from a fresh *S. cerevisiae pdr5*Δ*::URA3* culture were spread on synthetic complete medium plates containing 0.05% 5FOA and incubated at 30°C for 5 days. Colonies were streaked for isolation on YPD plates, tested by spot assay for growth on DOB-ura and 5FOA media, and their RNA analyzed as described above.

Results

PRODIGE method

PCR primers were designed to generate a disruption cassette that, following recombination, would precisely replace the coding sequence of the targeted gene with the coding sequence of the marker (Fig. 1a). In contrast to the conventional gene disruption methods, expression of the promoter-less marker gene (i.e., colony formation on selection plates) should be largely limited to cells in which the desired homologous recombination occurred, thus expressing the marker from the targeted gene's promoter. Consequently, while the yield of transfor-

Table 1 PCR primers used in this study

Primer[a]	Location[b]	Sequence (5′–3′)[c]
CgSLT2-ScURA3F	−58	*ATACAACCAGGTATCTACAACTTTAAAAGAAATTCTAGCGTATCCTACAAATCATATA*ATGTCGAAAGCTACATATAAGG
CgSLT2-ScURA3R	(+60)	*TACCTAATGGTTGTAGAAAAATATATATGTTATTTACTTTGCATGTCTCTCTATCTCCAA*TTAGTTTTGCTGGCCGCATC
CgSLT2uF	−368	GGGTCAAACTGGTAATACTCA
CgSLT2iR	+88	CATGGCCAATCTCTTTGATCA
CgLEM3-ScURA3F	−58	*ATAGCCGTTTTTTGTTCTGGAGTTTGACATAGAAAGGAGAGCAAGATAGGGAAAAAAA*ATGTCGAAAGCTACATATAAGG
CgLEM3-ScURA3R	(+60)	*ATGAACTGAAGAAATGCTTGTTTTCACCAGGCTAACTTAATCAGATTTAAAGAAGTTAAA*TTAGTTTTGCTGGCCGCATC
CgLEM3uF	−357	CGAACATACACCCACTATGC
CgLEM3iR	+74	GGCACATCTTCTTGGTACTC
CgPDR1-ScURA3F	−58	*GCCTTTTTTTTTAGAATATATTGGTAAAGTCATTCTTTAGCTACGTTATTGAGAGAAT*ATGTCGAAAGCTACATATAAGG
CgPDR1-ScURA3R	(+60)	*TGATTTTTCAGATTAAATATAAAATTATACAGGCTATGCACACTGTCTAAATTAATAGCA*TTAGTTTTGCTGGCCGCATC
CgPDR1uF	−424	GGCGTATTCATAGAATCCGAA
CgPDR1iR	+1672	CCATAGTATTCGTCGAGAGCA
CgADE2-TRP1F	−40	*TGTTACCAACGATACAGGTTTATTTTGCTTACGAATAATA*ATGTCTGTTATTAATTTCACAGG
CgADE2-TR1R	(+40)	*GAATTTCAAGCAAAGACTAACTGGTTTTATAGATGGTGCT*CTATTTCTTAGCATTTTTGACGA
CaLEM3-CaURA3F	−59	*TTCCAACCAATAGGACAGATACCAGGACACATAGCCCCTGTTTCATTATCCCCATAATC*ATGACAGTCAACACTAAGACC
CaLEM3-CaURA3R	(+57)	*TATTACAACACAATCTTTAATATAGACTTCAAAGAGAATTCATACTCAAAATAAACT*TTATAATTGGCCAGTTTTTTCA
CaLEM3uF	−217	CTACTGAACTGTTGCAACAGA
CaLEM3iR	+245	GCAATGGCAACTAACAACGG
CaLEM3-CaARG4F	−57	*CCAACCAATAGGACAGATACCAGGACACATAGCCCCTGTTTCATTATCCCCATAATC*ATGTCACAACAACAAGATAAACA
CaLEM3-CaARG4R	(+53)	*ACAACACAATCTTTAATATAGACTTCAAAGAGAATTCATA*CTCAAAATAAACTTTAACTTAAGATTGATTTTAAATTTTC
CaRHO1-ScURA3F	−58	*CCTCCCCTCTTCCCCTTTTAATAATACATCTATCAAATATAACATATAAACTTACATA*ATGTCGAAAGCTACATATAAGG
CaRHO1-ScURA3R	(+60)	*AGCTTTTGCTATTGCTAGTGCTTCTTCTTCTTCTTGTTCTTTTAGTTGTTGTTTCAT*TTAGTTTTGCTGGCCGCATC
CaRHO1uF	−164	GTTCCCTCTTTCTCTTTCTCT
CaRHO1dR	+740	CCACAACTCATAAGAGGGCT
CaURA3iR	+363	TGGTAATATCTGCCCAACTAC
CaARG4iR	+232	GTCTGATTTGTTCTAATCCTTG
ScPDR5-ScURA3F	−57	*AAATTAAAGACCCTTTTAAGTTTTCGTATCCGCTCGTTCGAAAGACTTTAGACAAAA*ATGTCGAAAGCTACATATAAGGA
ScPDR5-ScURA3R	(+59)	*TGTTTATTAAAAAAGTCCATCTTGGTAAGTTTCTTTTCTTAACCAAATTCAAAATTCTA*TTAGTTTTGCTGGCCGCATCT
ScPDR5uF	−255	AAACAAGGCCTCTCCTATACA
ScPDR5iR	+140	GCCAGTTTTTGGATTCGAGC
ScURA3iR	+55	CAGCAACAGGACTAGGATGAG
ScURA3iR2	+199	CAGTCAAGATATCCACATGTG

[a] See Fig. 1 for general diagram of primer location

[b] Location in nucleotides of 5′ end relative to the start codon of targeted gene or marker gene (for PRODIGE R primers, relative to stop codon of targeted gene)

[c] For PRODIGE primers, homology regions are italicized; start and stop codons of marker gene are underlined

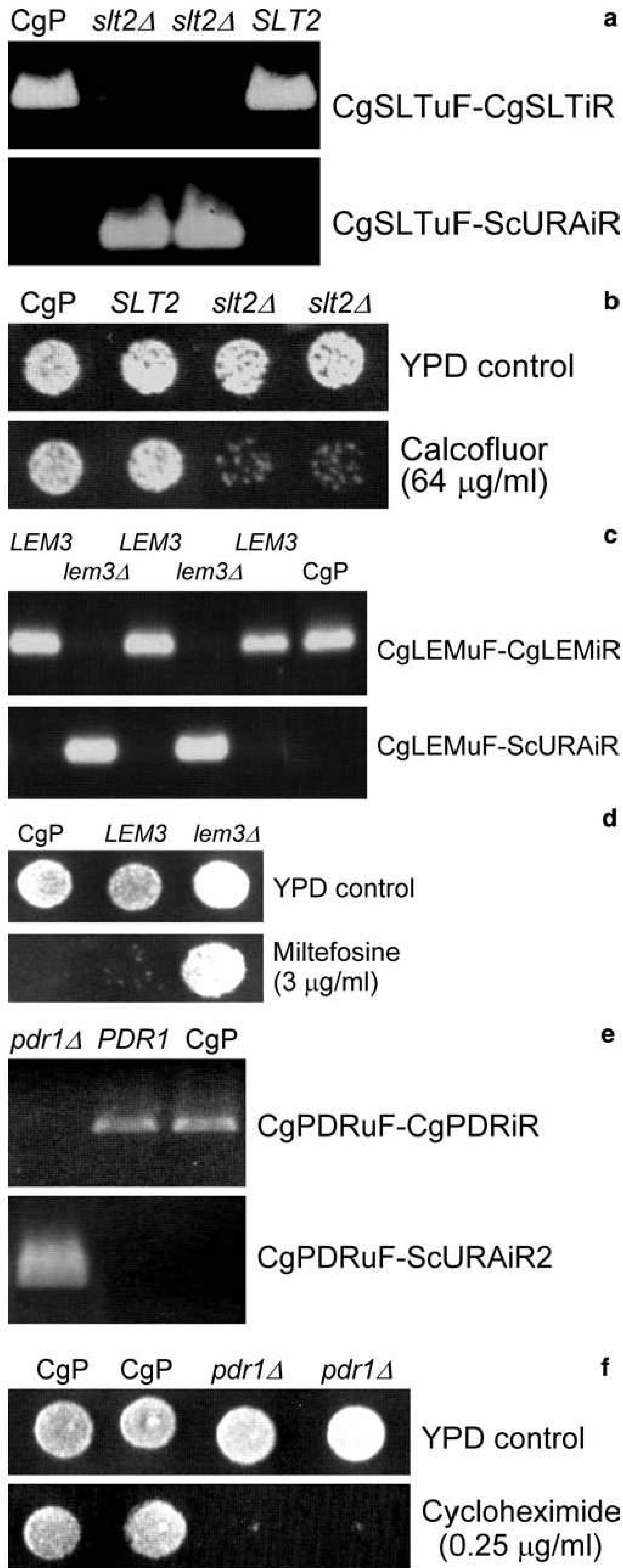

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Fig. 2 Representative PCR confirmation gels and drug sensitivity assays for *C. glabrata* gene disruptions. **a** *SLT2* disruption. Genomic DNAs were purified from isolated colonies and screened by PCR and gel electrophoresis. Loss of the CgSLT2uF-CgSLT2iR 456 bp product and formation of the CgSLT2uF-ScURA3iR 423 bp product (refer to Fig. 1b and Table 1 for primer locations and sequences) identified *slt2*Δ clones. CgP, *C. glabrata* parent strain; *SLT2*, a ura+ transformant that retained *SLT2* and hence represents a non-specific recombinant. **b** About 500 cells were spotted on YPD plates with or without calcofluor and incubated for 2 days. **c** *LEM3* disruption. Loss of the CgLEM3uF-CgLEM3iR 431 bp product and formation of the CgLEM3uF-ScURA3iR 412 bp product identified *lem3*Δ clones. **d** Cells were spotted on YPD with and without miltefosine and incubated for 2 days. **e** Disruption of *PDR1*. Loss of the CgPDR1uF-CgPDR1iR 2096 bp product and formation of the CgPDR1uF-ScURA3iR2 623 bp product identified *pdr1*Δ clones. **f** Cells were spotted on YPD with or without cycloheximide and incubated for 2 days

mants might be lower, the specificity (ratio of desired recombinants to total transformants) should be higher. Primers were typically 80 bases long, with 20–27 bases at the 3′ end corresponding to marker sequence termini and 53–60 bases at the 5′ end homologous to target gene flanking sequence. To minimize mutations to the marker coding sequence during PCR, a polymerase with relatively high fidelity was used along with moderate cycle numbers; the final extension was brief to minimize addition of non-templated residues. Following purification, the PCR products were used directly to transform yeast by standard methods.

C. glabrata SLT2, LEM3, and *PDR1* disruption

The haploid *C. glabrata* provided a stringent test of PRODIGE since, as noted above, short homology regions have yielded specificities of ≤ 2% (Cormack and Falkow 1999; Weig et al. 2001; our unpublished data). For our initial studies, we used the *S. cerevisiae URA3* coding sequence (derived from YEp24) as the selection marker which has previously been shown to be functional in *C. glabrata* (Kitada et al. 1995). Three different *C. glabrata ura3* host strains were used: BG14 (Cormack and Falkow 1999), ATCC 200989 (Kitada et al. 1995), and 8512-11 (a 5FOA-resistant derivative of clinical isolate 8512) (Vermitsky and Edlind 2004).

The *S. cerevisiae* gene *SLT2* encodes the MAP kinase of the cell integrity signaling pathway that confers protection from cell wall damaging agents such as chitin-binding agent calcofluor (Heinisch et al. 1999). A single, unambiguous *SLT2* homolog (CAGL0J00539g) was identified within the recently released *C. glabrata* genome sequence database (http://cbi.labri.fr/Genolevures/elt/CAGL) (Dujon et al. 2004). Using the PRODIGE method, *C. glabrata SLT2* was successfully disrupted in two strains with a specificity of 20% (3 of 15 and 5 of 25 transformants tested for strains BG14 and 200989, respectively). Representative PCR screening results with specific primer pairs (Table 1 and Fig. 1b) are presented (Fig. 2a). Specifically, the desired disruptants (labeled *slt2*Δ) show loss of the CgSLTuF-CgSLTiR product (i.e., product of *SLT2* upstream forward primer paired with *SLT2* internal reverse primer), while this product

Fig. 3 *C. glabrata ade2Δ* (*red or dark*) and non-specific (*white*) transformants on DOB-trp plate. PCR was used to confirm that *ade2* disruption was specific to red colonies (not shown).

was clearly observed with the *C. glabrata* parent (labeled CgP) and a representative non-specific transformant (labeled *SLT2*). Conversely, the desired disruptants generate the CgSLTuF-ScURAiR product (*SLT2* upstream forward primer paired with *URA3* internal reverse primer) indicative of *URA3* replacement of the *SLT2* coding sequence. The *C. glabrata slt2Δ* mutants demonstrated the expected phenotype of calcofluor hypersensitivity relative to parent strains and non-specific transformants; representative agar spot assay results are shown (Fig. 2b).

Using the same approach, *C. glabrata LEM3* (CAGL0D02442g) disruption mutants were generated in strain 200989 with a specificity of 31% (6 of 19 transformants tested); representative PCR screening results are shown (Fig. 2c). In *S. cerevisiae*, *LEM3* encodes a glycoprotein involved in the membrane translocation of phospholipids and alkylphosphocholine drugs such as miltefosine (Hanson et al. 2003). As in *S. cerevisiae*, *C. glabrata lem3Δ* mutants demonstrated miltefosine resistance (Fig. 2d).

The *C. glabrata PDR1* gene *PDR1* (CAGL0A00451g) was similarly disrupted (Fig. 2e). Specificity was only 5–8% in strains 200989 and BG14 (1 of 20 and 1 of 12 transformants, respectively) but 20% in strain 8512-11 (4 of 20). These results probably reflect the generally weak but variable expression of this transcription factor gene (Vermitsky and Edlind 2004). *C. glabrata PDR1* was previously implicated in transcriptional activation of the *CDR1* multidrug transporter gene (Vermitsky and Edlind 2004), and consistent with this *pdr1Δ* mutants were hypersensitive to cycloheximide (Fig. 2f).

C. glabrata ADE2 disruption: different markers, shorter homology, and comparison to conventional method

Disruption of *ADE2* in various yeast and molds results in the accumulation of a red-pigmented intermediate. We reasoned that this would provide a simple and sensitive screen, in lieu of PCR, with which to test different markers and shorter homology regions. Unpurified 60–63 nucleotide primer pairs with 40 nucleotides of *C. glabrata ADE2* homology were used to amplify *S. cerevisiae* marker coding sequences. In two independent experiments with *URA3*, transformation of *C. glabrata* 200989 generated about 3,000 ura+ colonies, but only 0.5% were red (not shown). In contrast, using the *TRP1* marker (from pRS414), about 800 trp+ colonies of varying size were obtained, of which 10% were red (Fig. 3). Attempts to similarly disrupt *ADE2* with the bacterial hygromycin resistance gene *hph* coding sequence (from pAG32, EUROSCARF; selection on 250–500 μg/ml hygromycin) were unsuccessful (not shown).

To directly compare PRODIGE with conventional PCR-product gene disruption, primers with identical 40 nucleotide *ADE2* homology regions were designed to amplify the *TRP1* gene including its promoter and polyadenylation regions as incorporated into pRS414 (281 bp upstream and 47 bp downstream of the coding sequence, respectively; not shown). Following amplification, PRODIGE and conventional cassettes were compared in parallel transformations of strain 200989. In two independent experiments (500–1,000 total transformants), PRODIGE yielded 5–10% red colonies while the conventional cassette yielded $<0.2\%$.

C. albicans LEM3 and *RHO1* disruption

We initially tested the PRODIGE method in diploid *C. albicans* by targeting its *LEM3* homolog (http://genolist.pasteur.fr/CandidaDB). The *C. albicans URA3* coding sequence (derived from pDDB57; Wilson et al. 1999) was used as the initial selection marker with host strain BWP17 (*ura3Δ/ura3Δ*, *arg4Δ/arg4Δ*, *his1Δ/his1Δ*; Wilson et al. 2000). Remarkably, *LEM3* allele disruption was achieved with a specificity of 95% (19 of 20 transformants). Representative PCR screening results for a heterozygous *LEM3/lem3Δ* mutant and its parent are shown in Fig. 4a. To directly compare PRODIGE with conventional PCR-product gene disruption, a disruption cassette was generated with identical *LEM3* homology regions but incorporating the *URA3* promoter and polyadenylation regions from pDDB57 (411 bp upstream and 275 bp downstream of the coding sequence, respectively; not shown). Comparable yields of ura+ colonies (25–30) were obtained in parallel transformations; however, PCR screening revealed

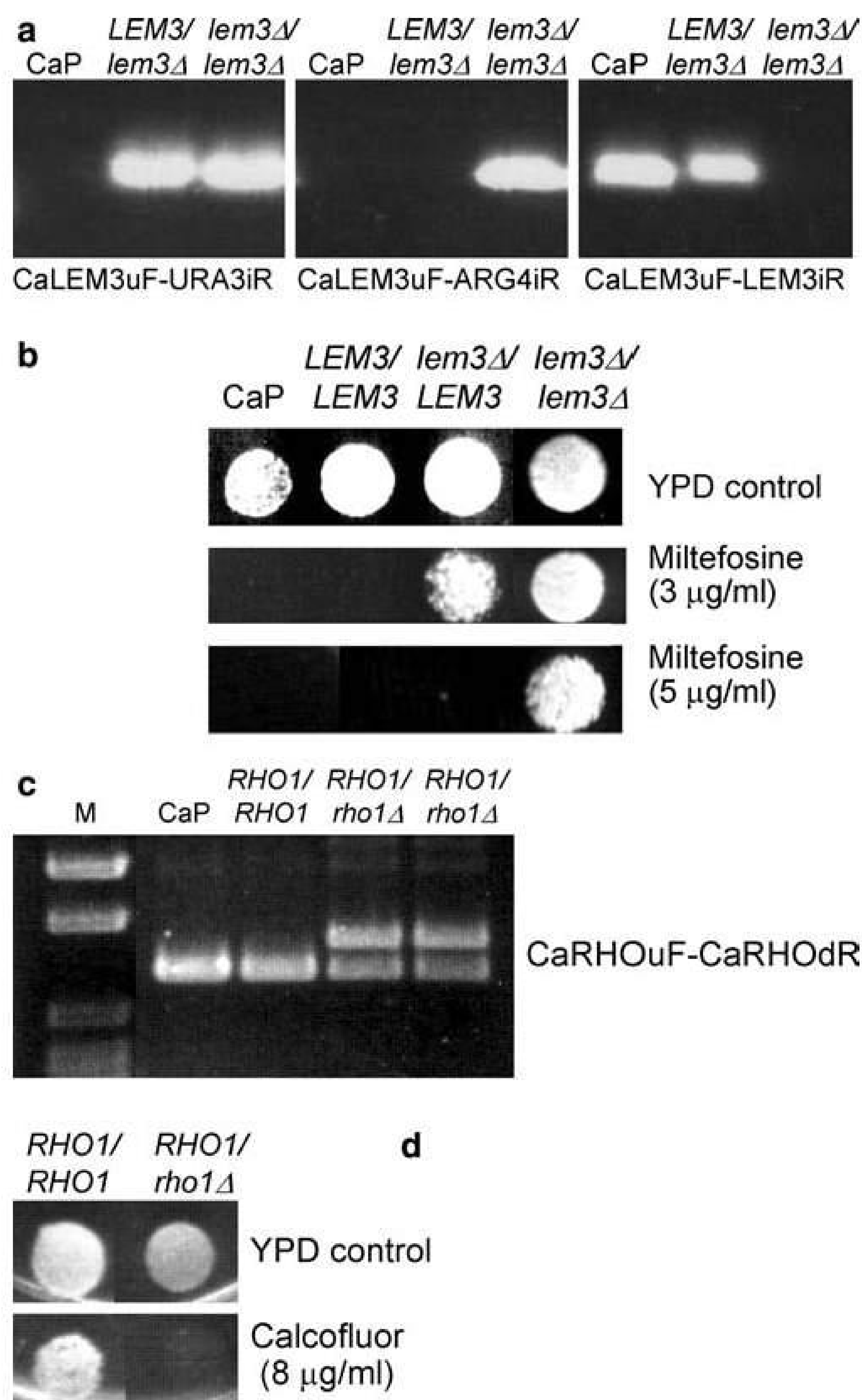

Fig. 4 Representative PCR confirmation gels and drug sensitivity assays for *C. albicans* gene disruptions. **a** Disruption of the first *LEM3* allele with *URA3* and the second with *ARG4*. Genomic DNAs were purified from isolated colonies and screened by PCR and gel electrophoresis. *C. albicans* lem3Δ::*URA3* disruption was identified based on formation of the CaLEM3uF-CaURA3iR (580 bp) product, and lem3D::ARG4 disruption based on formation of the CaLEM3uF-CaARG4iR (449 bp) product. Formation of the CaLEM3uF-CaLEM3iR (462 bp) product was observed in parent (CaP) and heterozygotes but not *lem3Δ/lem3Δ* transformants. **b** About 500 cells of the indicated strains were spotted on YPD with or without miltefosine and incubated 2–4 days. **c** *RHO1* disruption. Formation of 904 and 1111 bp CaRHO1uF-CaRHO1dR products identified *rho1Δ::URA3/RHO1* clones because the *S. cerevisiae URA3* coding sequence is 207 bp longer than the *C. albicans RHO1* coding sequence that it replaces. **d** Cells were spotted on YPD with or without calcofluor and incubated for 3 days

LEM3 disruption in 11 of 11 PRODIGE (100%) compared to 2 in 11(18%) conventional cassette transformants.

One approach to disrupting the second *C. albicans LEM3* allele would involve using the same *URA3* cassette and *LEM3/lem3Δ::URA3* cells that have been subjected to 5FOA-selection to regenerate *ura3* mutants. However, it is likely that recombination of the *URA3* marker into the *lem3Δ::URA3* allele would dominate over the desired recombination into the *LEM3* allele. The alternative approach involves using different markers. We initially tried *C. albicans HIS1* (from pFA-HIS1; Gola et al. 2003), but no transformants were obtained in several attempts (not shown). Using *C. albicans ARG4* (from pFA-ARG4), 79% (11 of 14) of the transformants on medium lacking both uridine and arginine showed disruption of both *LEM3* alleles (representative result in Fig. 4a). As with *C. glabrata lem3Δ* (above), the *C. albicans* heterozygous and homozygous *lem3Δ* mutants demonstrated miltefosine resistance, in proportion to gene copy number (Fig. 4b).

RHO1 is an essential gene in *C. albicans* and other yeast that functions in cell integrity signaling upstream of the MAP kinase pathway (Heinisch et al. 1999; Smith et al. 2002). For this experiment, we used the *S. cerevisiae URA3* coding sequence to test the general feasibility of using heterologous selection markers. This could prove useful in future studies of fungal species for which a cloned marker gene is unavailable, or in any strain where the marker is mutated but not deleted and hence remains a recombination target. Success seemed likely since marker expression in the PRODIGE method does not require recognition of a heterologous promoter. Indeed, 80% of transformants tested (8 of 10) demonstrated specific disruption of a *RHO1* allele (Fig. 4c). Their growth on ura-plates was, however, very slow. *S. cerevisiae URA3* includes one CTG codon which would be translated as Ser rather than Leu in *C. albicans* (Santos and Tuite 1995), and this probably accounts for the slow growth since mutant and parent grew comparably on YPD. As expected, *rho1Δ /RHO1* mutants were calcofluor hypersensitive (Fig. 4d).

S. cerevisiae PDR5 disruption: effects of variable marker expression

Expression of the *S. cerevisiae PDR5* multidrug transporter gene is mediated by the *PDR1* and *PDR3* transcriptional activators, which upregulate *PDR5* in response to drug treatment and other stresses (Kolaczkowska and Goffeau 1999). Their double disruption results in greatly reduced basal and drug-induced *PDR* expression (Fig. 5a). Thus, *pdr1Δ pdr3Δ* mutant (nA1Δ 3Δ) and *PDR1 PDR3* wild type (IPY36) strains were used to test the effects of transcriptional expression on PRODIGE-mediated *PDR5* disruption with a *URA3* marker. With the *pdr1Δ pdr3Δ* mutant, there were clear differences in the yield of transformants on ura-plates (tenfold lower) and growth rate in ura-broth (ninefold slower); nevertheless, specificity of *PDR5* disruption was ≥75% in both cases (Table 2). As expected, transformant sensitivity to 5FOA was proportional to the levels of *PDR5* promoter-mediated *URA3* expression (Table 2).

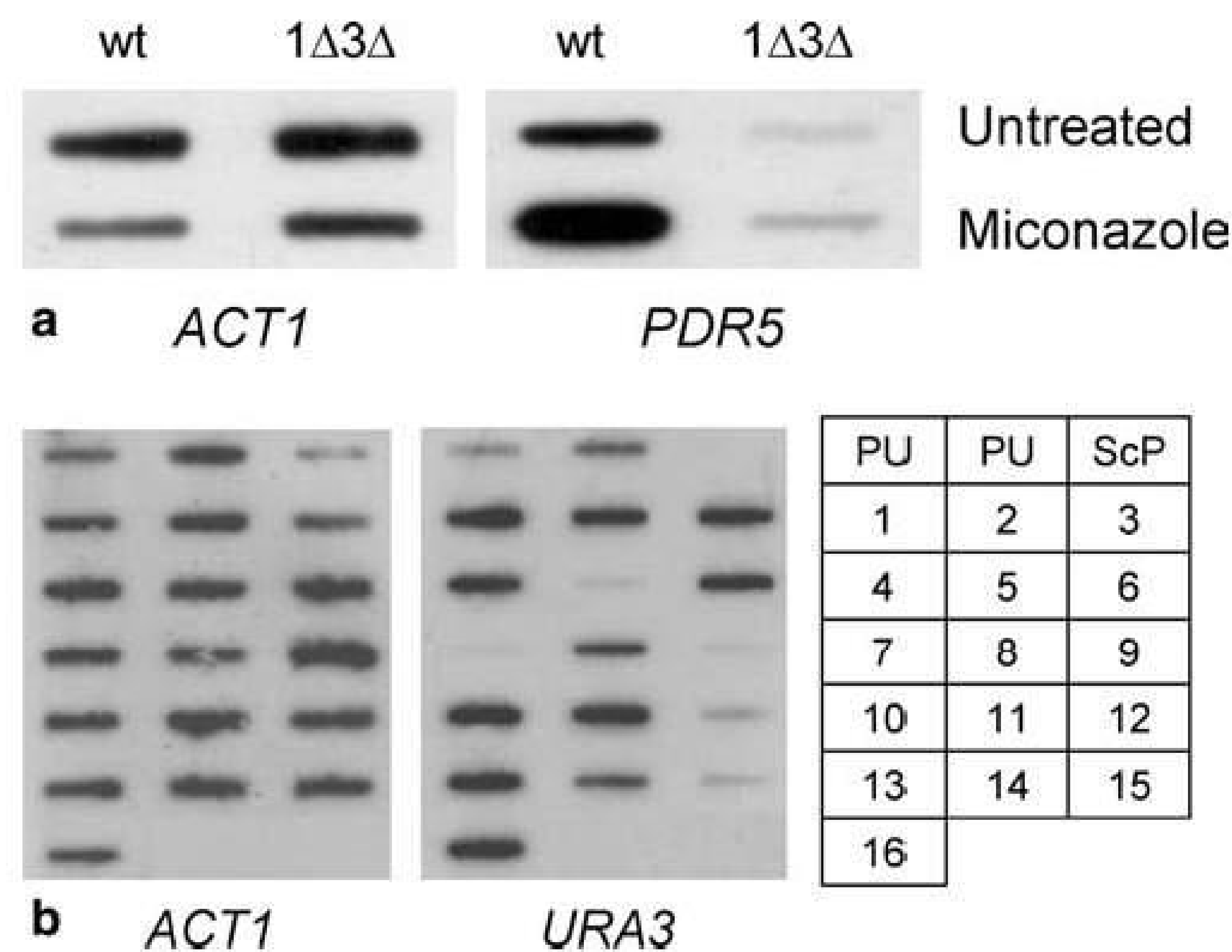

Fig. 5 **a** RNA slot blots comparing *S. cerevisiae PDR5* expression in wild-type *PDR1 PDR3* (wt) and *pdr1Δ pdr3Δ* (1Δ 3Δ) cultures, with or without miconazole treatment (15 µg/ml for 20 min). *ACT1* serves as loading control. **b** RNA slot blots examining *PDR5* promoter-dependent *URA3* expression in *S. cerevisiae pdr5Δ::URA3* cultures (PU) and 5FOA-resistant mutants (1–16); mutants 5, 7, 9, 12, and 15 show 8–10-fold decreased expression. Expression of *URA3* RNA in the *S. cerevisiae ura3-52* parent IPY36 (ScP) was not detected

S. cerevisiae pdr5Δ::URA3: selection of regulatory mutants

Since the flanking regulatory elements of the disrupted gene remain intact in the PRODIGE method, we reasoned that 5FOA could be used to select regulatory mutants of that gene when the marker is *URA3*. With respect to *PDR5*, for example, these could be *cis* mutations (within the *PDR5* promoter) or *trans* mutations (within a transcription factor gene such as *PDR1* or *PDR3*). The *pdr5Δ::URA3* strain constructed above (4×10^7 cells) was plated on 0.05% 5FOA-containing medium; after 5 days, 16 mutant colonies were observed. As shown by RNA hybridization (Fig. 5b), 5 of these 16 mutants demonstrated eight to tenfold reduced *URA3* expression, consistent with a regulatory mutation. All of these five were confirmed to be ura- (not shown). Of the remaining 5FOA-resistant mutants, six remained ura+, which is consistent with a mutation blocking 5FOA uptake, and five were ura- but retained full *URA3* expression consistent with a *URA3* coding sequence mutation.

Table 2 Effects of *pdr1Δ pdr3Δ* mutation on PRODIGE-mediated *PDR5* disruption in *S. cerevisiae*, and properties of transformants

	S. cerevisiae strain IPY36	nA1Δ 3Δ
Relevant genotype	*PDR1 PDR3*	*pdr1Δ pdr3Δ*
Transformants/µg DNA	29	3
Colony formation (h)	48–72	≥ 120
Relative growth (ura-/SC)	1.0	0.11
Specificity (*pdr5Δ*/total)	6/8 (75%)	3/3 (100%)
5-FOA sensitivity (MIC)	0.05%	≥ 0.4%

Discussion

Our initial goal in developing PRODIGE was to modify conventional PCR product-based gene disruption as widely applied to *S. cerevisiae* such that it could be usefully applied to *C. glabrata*. In this important opportunistic pathogen, the specificity of conventional gene disruption methods employing flanking homology regions ≤ 100 bp is ≤ 2% (Cormack and Falkow 1999; Weig et al. 2001; our unpublished data). With such low specificity, extensive screening is required to distinguish random integrants from desired disruptants. With PRODIGE, the flanking regulatory regions of the marker are eliminated such that marker expression (i.e., colony formation) should be limited to the desired recombinants in which the marker coding sequence precisely replaces the targeted gene coding sequence. This simple modification yielded averaged specificities of 11, 20, and 31% for three *C. glabrata* genes tested. Remarkably, when applied to *C. albicans*, PRODIGE yielded specificities of 79–95%, a significant improvement over conventional PCR-based methods with comparable (60–75 bp) homology regions (Wilson et al. 1999, 2000; Gola et al. 2003). We anticipate that PRODIGE can be applied broadly within the fungal kingdom and quite possibly beyond, and it should be amenable to high-throughput gene disruption projects. Two different nutritional markers were shown to be PRODIGE-compatible in *C. glabrata* (*URA3* and *TRP1*) and *C. albicans* (*URA3* and *ARG4*), while the hygromycin-resistance gene *hph* did not show any promise.

A recognized potential limitation of PRODIGE is that it requires a minimal level of promoter activity in the targeted gene to permit marker expression. This was demonstrated in our studies of *PDR5* disruption with *URA3* in an *S. cerevisiae pdr1Δpdr3Δ* mutant: compared to the parent strain there were tenfold fewer colonies, and these formed only after extended incubation. It remains to be seen what fraction of genes are PRODIGE-refractory due to insufficient transcription. In our experience with five different genes and the *URA3* marker this was not an issue; the disruption specificity for *C. glabrata PDR1* was relatively low (5–11%, depending on strain) but still required only minimal screening for detection. Gene expression databases are now available for many organisms (e.g., http://db.yeastgenome.org/cgi-bin/expression/expressionConnection.pl); this data may be useful in predicting poor gene targets for the PRODIGE approach. A related concern is that variable expression of nutritional markers, particularly *URA3*, may compromise phenotypic comparisons of different PRODIGE mutants. This is dealt with by using adequately supplemented medium in vitro, but this would not be feasible in vivo. A similar concern applies to conventional gene disruption approaches using *URA3* (Staab and Sundstrom 2003). Drug resistance markers provide a potential solution to this problem.

One assumption behind PRODIGE is that 60 bp of flanking homology incorporated into the disruption cassette are insufficient to confer transcription of the marker. We are not aware of any yeast genes that do not fit this assumption, i.e., have functional promoters within 60 bp of the start codon. Where this is an issue, it can be addressed by reducing the homology regions; our studies of *C. glabrata ADE2* disruption with the *TRP1* marker suggest that 40 bp homology regions may be adequate, which has the additional benefit of significantly reducing primer cost (by reducing both length and the need for purification). Further studies of homology length, marker, and target gene expression as variables in the PRODIGE method are needed.

A relatively minor advantage of PRODIGE versus conventional PCR product-mediated gene disruption is that the amplification products are about 500 or more bp shorter in the absence of marker promoter and polyadenylation regions. This can enhance the yield of the PCR reaction, an important consideration since some cells transform poorly and require large amounts of PCR product. Also, PRODIGE is more amenable to the use of heterologous markers. For example, we successfully used the *S. cerevisiae URA3* coding sequence to delete both *C. glabrata* and *C. albicans* genes. With conventional disruption methods this can be difficult because the less conserved flanking regulatory regions tend to confer species specificity. A heterologous marker is useful in host strains where the marker gene is not fully disrupted and consequently represents a site for undesired recombination events, or "marker reversion". The heterologous marker would be less likely to undergo such events because of sequence differences.

Extension of the PRODIGE method to the isolation of regulatory mutations appears to have much potential. The 5FOA-resistant *S. cerevisiae pdr5Δ::URA3* mutants that demonstrated decreased *URA3* expression are in the process of being characterized (K. Henry, unpublished data). Similarly, we are currently applying PRODIGE with *URA3* and 5FOA selection to the isolation of *C. glabrata* regulatory mutants.

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