A PCR-based strategy to generate integrative targeting alleles with large regions of homology

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***Cryptococcus neoformans* is an opportunistic fungal pathogen with a defined sexual cycle for which genetic and molecular techniques are well developed. The entire genome sequence of one *C. neoformans* strain is nearing completion. The efficient use of this sequence is dependent upon the development of methods to perform more rapid genetic analysis including gene-disruption techniques. A modified PCR overlap technique to generate targeting constructs for gene disruption that contain large regions of gene homology is described. This technique was used to disrupt or delete more than a dozen genes with efficiencies comparable to those previously reported using cloning technology to generate targeting constructs. Moreover, it is shown that disruptions can be made using this technique in a variety of strain backgrounds, including the pathogenic serotype A isolate H99 and recently characterized stable diploid strains. In combination with the availability of the complete genomic sequence, this gene-disruption technique should pave the way for higher throughput genetic analysis of this important pathogenic fungus.**

INTRODUCTION

Cryptococcus neoformans is a basidiomycetous fungus that has been recognized as a human pathogen for more than a century (Casadevall & Perfect, 1998; Sanfelice, 1894). *C. neoformans* mainly infects immunocompromised individuals, and disease typically manifests as meningoencephalitis (Casadevall & Perfect, 1998). With the relative increase in recent years in the numbers of immunocompromised patients due to AIDS, cancer chemotherapy, transplant-rejection suppression therapy, and other factors, the incidence of opportunistic fungal infections has risen (Mitchell & Perfect, 1995). *C. neoformans* infection results from inhalation of spores or desiccated yeast cells, which then spread via the bloodstream to infect the central nervous system and cause life-threatening meningoencephalitis.

In addition to being an important cause of disease in immunocompromised patients, *C. neoformans* is an excellent model of fungal pathogenesis. Several well-defined virulence factors include the polysaccharide capsule (which promotes intracellular survival in macrophages), the pigment melanin (which prevents oxidation by macrophages), the production of the enzymes urease and phospholipase B, and the ability to grow at 37 °C (Alspaugh et al., 2000a; Casadevall & Perfect, 1998; Chen et al., 2000; Cox et al., 2000, 2001; Cruz et al., 2000; Fox et al., 2001; Kwon-Chung & Bennett, 1992; Odom et al., 1997). Several animal models have been developed, including a mouse tail-vein injection model, rat and mouse inhalation models, and a rabbit meningitis model (Goldman et al., 1994; Kwon-Chung et al., 1982; Perfect et al., 1980). Importantly, *C. neoformans* is genetically tractable, with a well-characterized sexual cycle involving mating between two haploid mating types, *MAT***a** and *MAT*α (Alspaugh et al., 2000b; Kwon-Chung, 1975, 1976). Efficient transformation and gene-disruption protocols have been developed along with auxotrophic and dominant selectable markers and

overexpression plasmids (Davidson *et al.*, 2000; Edman & Kwon-Chung, 1990; Hua *et al.*, 2000; McDade & Cox, 2001; Sudarshan *et al.*, 1999; Toffaletti *et al.*, 1993). Furthermore, genetic analysis has been facilitated by the recent characterization of a stable diploid state, which allows identification and analysis of essential genes (Sia *et al.*, 2000). Finally, the entire genome sequence is nearing completion for the serotype D strain JEC21, and a sequencing project has been initiated for the clinical serotype A isolate H99 (Stanford, TIGR, UBC, Duke, Whitehead; reviewed by Heitman *et al.*, 1999). These advances will allow extensive comparative genomics experimentation between *C. neoformans* strains and other fungal genomes.

The efficient use of the complete genome sequence will require more rapid gene-function testing, a process that is limited by tedious construction of targeting alleles by cloning. PCR-generated targeting alleles have been employed for *Saccharomyces cerevisiae* and for the human pathogen *Candida albicans*, which has overcome the necessity for cloning and has allowed more high-throughput genetic analysis (Baudin *et al.*, 1993; Eberhardt & Hohmann, 1995; Lorenz *et al.*, 1995; Wach, 1996; Wach *et al.*, 1994; Wilson *et al.*, 1999). However, efficient homologous recombination in *C. neoformans* requires larger regions of homology, which has prevented the application of similar PCR-based strategies.

Here we present a modified application of a technique called PCR overlap to generate targeting alleles with larger regions of homology (Ho *et al.*, 1989; Horton *et al.*, 1989). This technique can be used in any system where homologous recombination requires longer regions than those that can be incorporated into synthetic oligonucleotides, and will effectively eliminate the time-consuming process of searching for convenient restriction sites and cloning targeting alleles.

METHODS

Gene disruptions. To create the *ste11α* mutant strain, first the ~4·5 kb *STE11α* locus was amplified from MATα genomic DNA using primers JOHE5391 (GCTCGTTCTCCCCTGT-AC) and JOHE5392 (CTGCCGACGCCGTGTAAT) (R. C. Davidson and others, unpublished). The *ste11αΔ::URA5* disruption allele was constructed using PCR overlap (outlined in Fig. 1). In the first round, the 5′ end of the *STE11α* gene was amplified with primers JOHE5391 (primer 1) (GCTCGTTC-TCCCCTGTAC) and JOHE5306 (primer 3) (**GGTCGAGC-AACTTCCTC**ATTTACAGGGCTGTCCTG), the 3′ end of the *STE11α* gene with primers JOHE5307 (primer 4) (**CCAC-CTCCTGGAGGCAAG**ACAGGGATATCAAAGGCG) and JOHE5392 (primer 6) (CTGCCGACGCCGTGTAAT), and the *URA5* gene with primers JOHE5305 (primer 2) (CAGG-ACAGCCCTGTAAAT**GAGCGAAGTTGCTCGAAC**) and JOHE5308 (primer 5) (CGCCTTTGATATCCCTGT**CTT-GCCTCCAGGAGGTGG**). The bold text represents the fragment of the oligo corresponding to the selectable marker. The amplified products were run on a 0·6% agarose gel and extracted together using the Qiaquik column method (Qiagen). Primers JOHE5391 (no. 1) and JOHE5392 (no. 6)

were then used to overlap the three products to yield the 4·2 kb *ste11αΔ::URA5* disruption allele. The PCR product was gel-purified, extracted and directly introduced into the serotype D *ura5* strain JEC43 by biolistic transformation.

The *tor1Δ::URA5/TOR1* mutant strain was generated by PCR overlap using six primers as outlined (Fig. 1). The 5′ end of the *TOR1* gene was amplified with primers JOHE4337 (no. 1) (CACTGAGTTGGAGTATTCTAC) and JOHE5850 (no. 3) (**CATGGTCATAGCTGTTTCC**TCGCCAAAGACGTA-GAAGGC) and the 3′ end of *TOR1* with primers JOHE5851 (no. 4) (**ACTGGCCGTCGTTTTAC**GATAGACTCGAGG-ACGAGC) and JOHE4413 (no. 6) (GTCACCAAAGTAGT-GCTTTG) and the *URA5* selectable marker with primers JOHE5849 (no. 2) (GCCTTCTACGTCTTTGGCGA**GGA-AACAGCTATGACCATG**) and JOHE5852 (no. 5) (GCTC-GTCCTCGAGTCTATC**GTAAAACGACGGCCAGT**). The *URA5* fragment in this disruption was amplified from plasmid pRCD69, which contains the *C. neoformans* serotype D *URA5* sequence in the pCR2.1-TOPO vector, and the primers were designed to amplify from the vector sequence. The three amplified products were run on a 0·6% agarose gel and extracted together using the Qiaquik column method (Qiagen) and used as templates for the overlap reaction. Primers JOHE6209 (no. 1) and JOHE4413 (no. 6) were used to overlap the three first-round products to yield the *tor1Δ::URA5* allele. The PCR product was gel-purified, extracted and transformed directly into the *C. neoformans* serotype D diploid strain RAS008 by biolistic transformation.

The *mpk1Δ::URA5* mutant strain was generated by PCR overlap using six primers as outlined (Fig. 1). The 5′ end of the *MPK1* gene was amplified with primers JOHE7288 (no. 1) (ACTAGGCGTGCCATTGTTTAC) and JOHE7418 (no. 3) (**GGTCGAGCAACTTCGCTC**AGGATTGCGTGCCGG-ACAGTG), the 3′ of *MPK1* with primers JOHE7419 (no. 4) (**CCACCTCCTGGAGGCAAG**CACGATGATTATTAG-TCTTGC) and JOHE7289 (no. 6) (GCGGACTGGGCA-GGAGAAGC), and the *URA5* selectable marker with primers JOHE7417 (no. 2) (CACTGTCCGGCACGCAATCCT**GA-GCGAAGTTGCTCGACC**) and JOHE7420 (no. 5) (GCAA-GACTAATAATCATCGTG**CTTGCCTCCAGGAGGT-GG**). The three amplified products were run on a 0·6% agarose gel and extracted together using the Qiaquik column method (Qiagen) and were used as templates for the overlap reaction. Primers JOHE7288 (no. 1) and JOHE7289 (no. 6) were used to overlap the three first-round products to yield the *mpk1Δ::URA5* disruption allele. This amplified product was gel-purified, extracted and directly transformed into the serotype D *ura5* strain JEC43 by biolistic transformation.

Overlap primer design. Overlap oligos were approximately 35–40 bp in length. Primers were obtained from Integrated DNA Technology and were not PAGE-purified. In the *STE11α* and *MPK1* deletions, the portion of the oligo corresponding to the selectable marker flanked the *C. neoformans URA5* gene. In the *TOR1* disruption, the portion of the oligo corresponding to the selectable marker was designed to amplify from a plasmid containing *URA5*. This was done so that any selectable marker cloned into the vector could be easily inserted into the disruption cassette.

The sequence of primer 3 was designed to be completely complementary to the sequence of primer 2, and the sequence of primer 4 was complementary to the sequence of primer 5 as outlined in Fig. 1. This strategy generates fragments with approximately 40 bp of overlap for the final PCR reaction. The 3′ ends of primer 3 and primer 4 were designed so that the
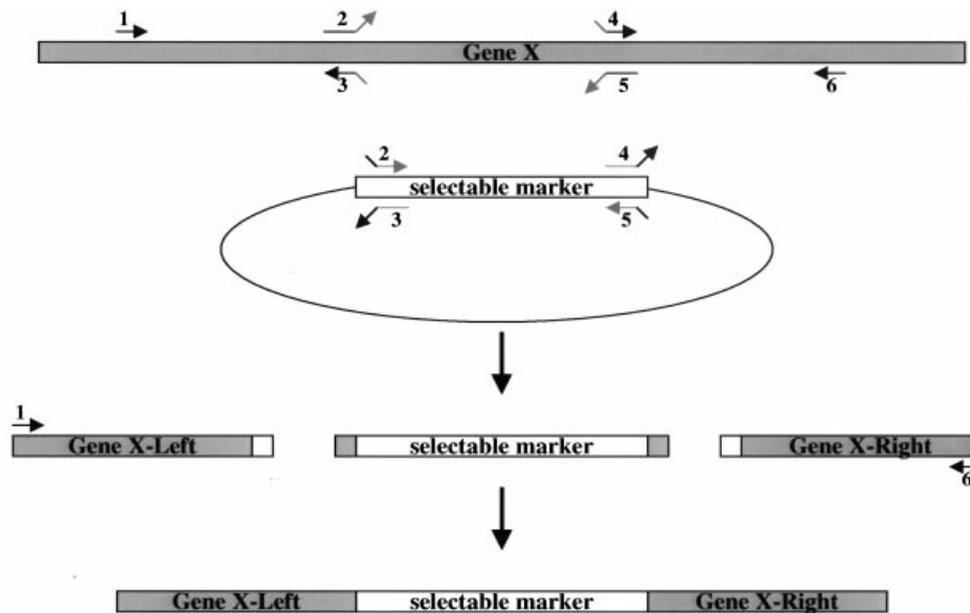
Fig. 1. The PCR overlap procedure. A gene deletion allele is constructed by PCR as follows: the 5′ end of the gene to be deleted (Gene X) is amplified with primers 1 and 3 to generate fragment Gene X-Left, the 3′ end of Gene X is amplified with primers 4 and 6 to generate fragment Gene X-Right, and the selectable marker is amplified with primers 2 and 5. The amplified products are then used as templates for an overlap reaction using primers 1 and 6. Primers 2 and 3 and primers 4 and 5, respectively, are complementary to one another and will therefore allow primers 1 and 6 to overlap the three first-round products into a linear PCR-amplified GeneX::selectable marker deletion allele.

melting temperature of the oligo fragment corresponding to the targeted gene was similar to the melting temperatures of primers 1 and 6, respectively.

Transformations. Biolistic transformations were performed by the method previously described by Toffaletti & Perfect (1994) using a Bio-Rad model PDS-1000/He biolistic particle delivery system. Transformants were selected on defined medium (Sherman, 1991) lacking uracil and containing 1 M sorbitol as an osmotic stabilizer.

PCR amplification. All PCR amplifications were performed using a Perkin-Elmer Applied Biosystems 9600 thermocycler with Extaq polymerase (Takara). The initial amplification reactions consisted of 35 cycles of 10–30 s at 95 °C, 10–30 s at 55 °C and 1 min kb^{-1} at 72 °C with an initial denaturation of 2 min at 95 °C and a final extension of 5 min at 72 °C. Importantly, denaturation and annealing times were limited to 10–15 s during the final overlap amplification, which reproducibly increases the yield of specific overlap products.

The first-round PCR of the *STE11α* disruption consisted of an initial denaturation of 2 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 15 s at 53 °C and 1·5 min at 72 °C, and was completed with a final extension of 5 min at 72 °C. The final round of PCR for the *STE11α* gene disruption consisted of an initial denaturation of 2 min at 95 °C, followed by 35 cycles of 15 s at 95 °C, 15 s at 53 °C and 4·5 min at 72 °C, and concluded with a final extension of 5 min at 72 °C.

In the final PCR, the three PCR products generated in the first PCR were added in roughly equimolar amounts. The quantity of these products yielding the most efficient overlap in the final PCR varied between constructs, so a gradient of first-round products was added to the reaction (generally 1 ng, 10 ng and 50 ng total template) to obtain the most efficient overlap PCR.

The PCR samples were visualized using standard DNA electrophoretic techniques (Sambrook *et al.*, 1989).

Southern blot analysis. Genomic DNA was isolated from JEC21 and JEC20 wild-type, *ste11α* and *mpk1* mutant strains by the method of Pitkin *et al.* (1996). Twenty micrograms genomic DNA was digested with the appropriate enzymes and electrophoresed on a 0·8 % TBE agarose gel. Transfer, hybridization and autoradiography were performed as described by Sambrook *et al.* (1989). Fragments of the *STE11α* and *MPK1* genomic ORFs were used as probes for Southern blot hybridization, using [α-^{32}P]dCTP (Amersham) and the Prime-It II random primed labelling kit (Stratagene).

RESULTS

Targeted disruption of the *STE11α* gene

The *STE11α* gene encodes a mating type-specific MEK kinase homologue that is related to the Ste11 kinase of *S. cerevisiae*, a component of the pheromone-sensing mitogen-acitvated protein (MAP) kinase cascade. The *C. neoformans STE11α* gene was identified by its location in the *MATα* locus adjacent to the first pheromone gene discovered in *C. neoformans* by Moore & Edman (Clarke *et al.*, 2001; Moore & Edman, 1993). To perform epistasis analysis with other members of this MAP kinase cascade, we made a *ste11α* mutant strain by homologous integration of a disruption allele. We used PCR to generate a *ste11α::URA5* disruption allele. Six oligonucleotide primers (numbered 1–6) were directed against the *STE11α* ORF and the *URA5* selectable marker (strategy outlined in Fig. 1). Separate PCR
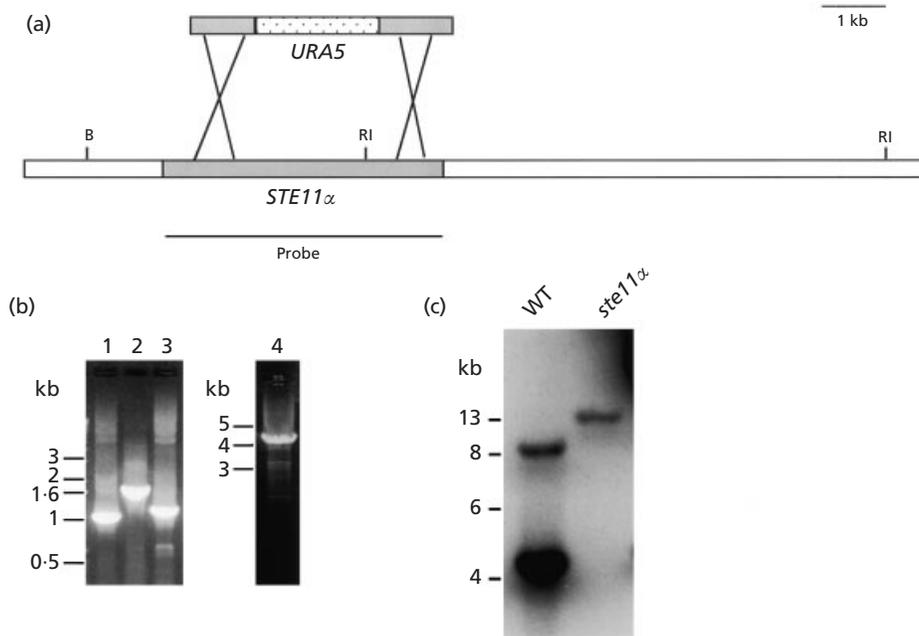
Fig. 2. Deletion of the *STE11α* gene. (a) The *STE11α* gene was deleted by replacing a portion of the *STE11α* ORF with the *URA5* selectable marker by PCR overlap deleting 2·2 kb of *STE11α*. (b) The 5′ region of *STE11α* (lane 1), the *URA5* selectable marker (lane 2), and the 3′ region of *STE11α* (lane 3) were amplified as described (Methods). The three products were used as templates to amplify the *ste11α::URA5* deletion allele (lane 4). (c) Southern analysis was performed to confirm the gene deletion. Genomic DNA from wild-type *MATα* (WT) and the *ste11α* mutant strain was digested with *Bam*HI (B) and *Eco*RI (RI), subjected to Southern blot analysis, and probed with a 4 kb PCR fragment of the *STE11α* gene. Positions of DNA size markers are shown on the left.

amplification with these primers yielded three over-lapping products: the 5′ end of the *STE11α* gene, the *URA5* selectable marker, and the 3′ end of the *STE11α* gene (Fig. 2a, b, lanes 1–3). In a second round of amplification, the three products were used as templates to generate a single linear disruption allele that con-tained the *URA5* gene flanked by portions of the *STE11α* gene (Fig. 2b, lane 4). This linear PCR product was directly transformed into a serotype D *MATα ura5* strain of *C. neoformans* (JEC43) by biolistic trans-formation, and Ura⁺ colonies were selected. Screening by PCR amplification revealed three mutant strains out of 48 independent Ura⁺ colonies (6·3%) (Table 1), which was confirmed by Southern blot analysis (Fig. 2c). Each of the *ste11α* mutant strains exhibited the same level of sterility in a cross with a wild-type *MATa* strain, and this was consistent with results obtained with mutants lacking other members of the MAP kinase cascade (R. C. Davidson & J. Héitman, unpublished).

Targeted disruption of the *TOR1* gene

The *TOR1* gene encodes a homologue of the Tor kinases of *S. cerevisiae*. To obtain functional infor-mation about this gene, we took a gene-deletion approach. The gene was originally identified in serotype A (Cruz *et al.*, 1999), and the serotype D sequence was then partially assembled from the Stanford and TIGR databases by comparison of serotype A and serotype D

sequences. Six primers (numbered 1–6) were constructed to amplify the 5′ and 3′ portions of the *TOR1* gene and the selectable marker, *URA5* (Fig. 3a, b, lanes 1–3). These three products and primers 1 and 6 were used to amplify a single linear disruption allele that consisted of *URA5* flanked by ∼1 kb of *TOR1* gene sequence on each side (Fig. 3b, lane 4). The linear overlap PCR product was gel-purified and used directly for trans-formation into a haploid recipient strain of *C. neo-formans*. No homologous recombination events were obtained out of approximately 200 Ura⁺ isolates screened, indicating that *TOR1* might be an essential gene in *C. neoformans*.

To establish whether *TOR1* is essential or not, we sought to isolate *tor1/TOR1* heterozygous mutants using the recently discovered stable, congenic diploid strains of *C. neoformans* (Sia *et al.*, 2000). A *ura5/ura5* diploid strain of *C. neoformans* (RAS008) was trans-formed with the linear *tor1Δ::URA5* PCR-generated disruption allele, and Ura⁺ transformants were selected. Using PCR amplification and digestion with the *Sal*I restriction enzyme, which specifically cleaves the mutant allele but not the wild-type at a pair of recognition sites present in the *URA5* gene (Fig. 3c), one *tor1::URA5/ TOR1* strain was identified out of 56 Ura⁺ transformants screened (1·8%) (Table 1). These results indicate that PCR-generated disruption constructs can be used to target genes in the laboratory-constructed congenic

Table 1. Genes disrupted using PCR-generated targeting alleles

Gene	Strain (genotype)	Frequency* (%)	Homology†
ste11α∆::URA5	JEC43 (Sero D *MATα ura5*)	2	1040…1180
tor1∆::URA5	RAS008 (Sero D *MATa/α ura5/ura5*)	2	1222…1030
mpk1∆::URA5	JEC43 (Sero D *MATα ura5*)	10	628…835
ste12α∆::URA5	JEC155 (Sero D *ade2-27 ura5*)	10	1476…1661
tlk1∆::URA5	JEC34 (Sero D *MATa ura5*)	4	789…956
fhb1∆::URA5	JEC43 (Sero D *MATα ura5*)	33	1044…1296
fhb1∆::URA5	H99 5-FOAʳ (Sero A *MATα ura5*)	22	682…1113
gno1∆::NAT1	JEC34 (Sero D *MATa ura5*)	23‡	792…656
gno1∆::NAT1	H99 5-FOAʳ (Sero A *MATα ura5*)	77‡	549…656
prf1∆::URA5	JEC43 (Sero D *MATα ura5*)	87	800…361
pka2∆::URA5	JEC43 (Sero D *MATα ura5*)	3	530…860
sxi1α∆::URA5	JEC43 (Sero D *MATα ura5*)	10	1102…1007
sxi1α∆::URA5	RAS009 (Sero D *MATa/α ura5/ura5*)	4	1102…1007
sxi1α∆::NAT1	JEC43 (Sero D *MATα ura5*)	10	1103…1014
sxi1α∆::NAT1	RAS009 (Sero D *MATa/α ura5/ura5*)	ND	1103…1014
gpa1∆::URA5	H99 5-FOAʳ (Sero A *MATα ura5*)	12	650…540
crg1∆::URA5	PPW28 (Sero A *MATα ade2 sch9∆::ADE2 ura5*)	10	590…605

*Frequency refers to the homologous recombination frequency obtained. ND, Not determined.

†Homology refers to the amount of the 5′ (left) and 3′ (right) regions of each gene incorporated into the disruption alleles in base pairs.

‡In these cases, this is the frequency of homologous recombination in isolated stable transformants.
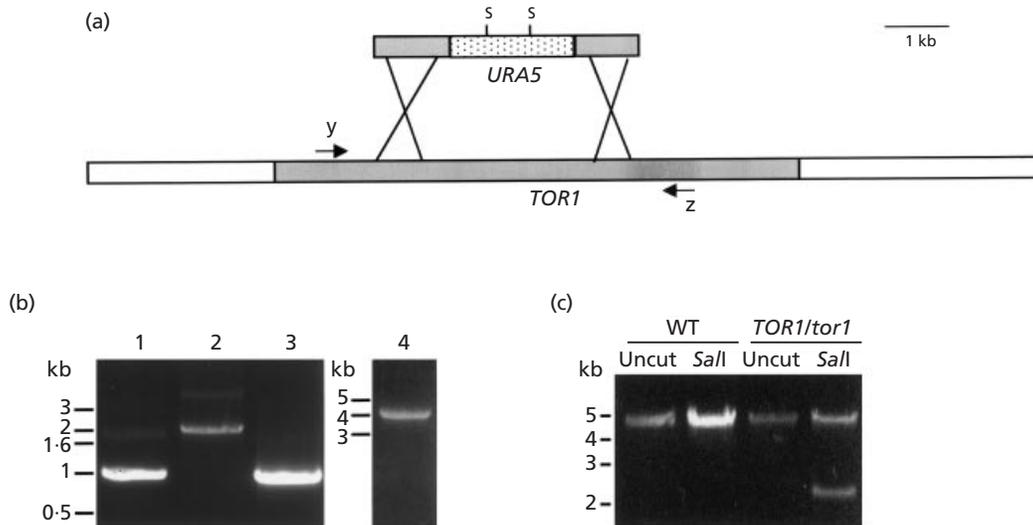
Fig. 3. Deletion of the *TOR1* gene. (a) The *TOR1* gene was deleted by replacing a portion of the *TOR1* ORF with the *URA5* selectable marker by PCR overlap. (b) The 5′ region of *TOR1* (lane 1), the *URA5* selectable marker (lane 2), and the 3′ region of *TOR1* (lane 3) were amplified as described (Methods). The three products were used as templates to amplify the *tor1::URA5* deletion allele (lane 4). (c) PCR analysis was performed to confirm the heterozygous gene deletion. Genomic DNA from wild-type *MATα* (WT) and the *tor1/TOR1* mutant strain was PCR-amplified with primers y and z and digested with *Sal*I (S), which specifically digests the mutant allele but not the wild-type allele. Two of the fragments generated by the digestion of the mutant disruption allele were approximately the same size and thus appear as a single 2·1 kb band. The smaller *Sal*I–*Sal*I fragment inside the *URA5* sequence of approximately 650 bp is not shown in this figure.

diploid strains, and provide evidence that *TOR1* is an essential gene, since it could be disrupted in a diploid, but not a haploid, strain of *C. neoformans*. We do note that gene disruption in the congenic serotype D diploid strains typically results in a lower frequency of homologous recombination (1–5%) than that commonly observed in haploid recipient strains (2–25% on average).
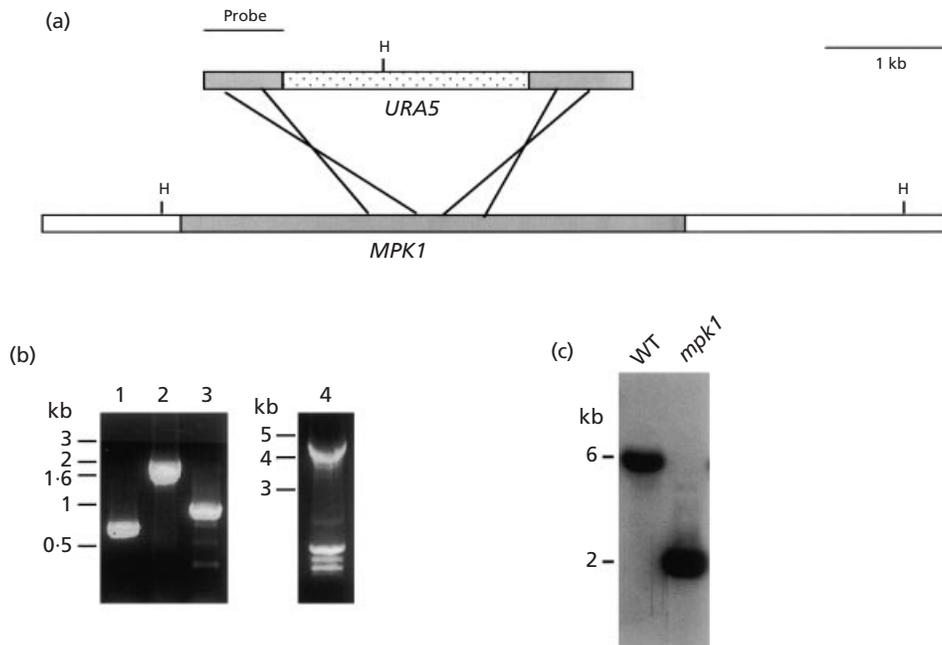
Fig. 4. Disruption of the *MPK1* gene. (a) The *MPK1* gene was disrupted by introducing the *URA5* selectable marker into the *MPK1* ORF by PCR overlap. (b) The 5′ region of *MPK1* (lane 1), the *URA5* selectable marker (lane 2), and the 3′ region of *MPK1* (lane 3) were amplified as described (Methods). The three products were used as templates to amplify the *mpk1*::*URA5* deletion allele (lane 4). (c) Southern analysis was performed to confirm the gene deletion. Genomic DNA from wild-type *MAT*α (WT) and the *mpk1* mutant strain was digested with *Hin*dIII (H), subjected to Southern blot analysis, and probed with a 1 kb PCR fragment of the 5′ end of the *MPK1* gene. Positions of DNA size markers are shown on the left.

Targeted disruption of the *MPK1* gene

The gene encoding the MAP kinase homologue Mpk1 was identified by comparative genomics using the Stanford *C. neoformans* genome sequence database (P. R. Kraus and others, unpublished). A complete sequence of the putative gene was assembled using the Stanford sequence as well as that from the TIGR *C. neoformans* sequencing project. To assess the function of this MAP kinase homologue, we generated an allele for gene disruption using PCR overlap. As described above, primers were directed against the 5′ and 3′ flanking regions of the *MPK1* gene and the *URA5* selectable marker, and three products were amplified (Fig. 4a, b, lanes 1–3). These products were overlapped into a single product with the portions of the *MPK1* gene flanking *URA5* (Fig. 4b, lane 4). The *mpk1*Δ::*URA5* PCR-generated disruption allele was transformed into a serotype D *ura5* strain of *C. neoformans* (JEC43), and Ura+ colonies were selected. Two *mpk1*Δ::*URA5* mutant strains were identified by PCR out of 20 screened (10 %) (Table 1), which was confirmed by a Southern blot (Fig. 4c).

Summary of results

Together, these results indicate that PCR overlap can be used to generate targeting alleles for gene disruption in *C. neoformans*. Successful PCR overlap amplification products have been obtained in each case for which it has been attempted (Table 1). However, in some cases, obtaining sufficient product for transformation has required manipulation of PCR parameters, particularly when transforming diploid strains, which typically require more transforming DNA (3–5 µg) to obtain transformants. For example, reducing the denaturation and annealing times to 15 s or less has increased product yield dramatically in several cases, particularly when using the dominant selectable marker *NAT1*. Additionally, to maximize product, it is important to maintain relatively equimolar amounts of the templates in the overlap reaction. In all, we have obtained 17 gene disruptions using this PCR overlap technique to generate targeting alleles. Thus, we show that the PCR overlap technique is generally applicable and can be used with a variety of recipient strains as well as with both auxotrophic and dominant selectable markers (Table 1).

DISCUSSION

C. neoformans is an important human pathogenic fungus. As a genetically tractable organism with well-developed molecular biology and animal models, and a nearly completed genomic sequence that is publicly available, *C. neoformans* is an excellent model for the study of fungal pathogenesis (Casadevall & Perfect, 1998). However, efficient use of the sequence data

requires implementation of improved molecular techniques such as gene disruption. In many model systems, PCR has replaced cloning as a faster, more efficient method of generating disruption alleles. Generation of disruption constructs in *S. cerevisiae* is performed almost exclusively by the technique involving large synthetic oligonucleotides that contain 40 bp of homology to the gene of interest followed by 20 bp of homology to the selectable marker (Eberhardt & Hohmann, 1995; Lorenz *et al.*, 1995; Wach, 1996; Wach *et al.*, 1994). The selectable marker is then amplified and the PCR product used to directly transform and generate the mutant strain (Tong *et al.*, 2001). This technique was also recently implemented for producing mutant strains in the human pathogen *Candida albicans* (Wilson *et al.*, 1999). However, in *Cryptococcus neoformans*, a larger region of homology is required for integration and thus cannot be incorporated into synthetic oligonucleotides.

We describe a modified use of the PCR overlap technique originally described by Ho *et al.* (1989) and Horton *et al.* (1989) to generate disruption constructs without the need for cloning. This method can be used to generate both partial and complete deletions of the targeted gene's ORF. Using this method, we have disrupted three genes, *STE11α* (encoding a MAP kinase kinase kinase homologue), *TOR1* (encoding a Tor kinase homologue) and *MPK1* (encoding a MAP kinase homologue), at efficiencies consistent with previous studies (Davidson *et al.*, 2000). We also report the disruption of 11 other genes using the same method and a variety of recipient strains, including the recently described serotype D diploids (Sia *et al.*, 2000). Moreover, both the auxotrophic *URA5* and dominant *NAT1* selectable markers were used, which allows generation of double mutant strains or the use of prototrophic strains. In all cases, the efficiency of targeted integration was in the range 2–10 % or higher, consistent with previous studies using biolistic transformation in *C. neoformans* (Alspaugh *et al.*, 1997; Davidson *et al.*, 2000; Toffaletti *et al.*, 1993; Wang *et al.*, 2000). These findings indicate that generation of constructs by two rounds of PCR does not seem to inhibit homologous recombination significantly.

Additional screening tests can make the PCR-disruption method even more efficient. Jennifer Lodge and coworkers recently found that a significant proportion of initial transformants are unstable, in accord with earlier results (Edman & Kwon-Chung, 1990), and by implementing an initial screening step for stable transformants they found that the efficiency of homologous targeting can be increased (Nelson *et al.*, 2002). We have confirmed these observations in the background of our PCR-based approach to gene disruption and found that approximately 33 % of transformants are stable. In the case of the *gno1*::*NAT1* disruption in strain JEC34, the frequency of homologous integration was increased from 10 to 23 %, and, in the case of the *gno1*::*NAT1* disruption in strain H99, from 28 to 77 %, by first screening for stable transformants. Thus, implementing this important advance of Lodge and coworkers, together with the PCR overlap approaches described here,

should result in even more efficacious gene-disruption frequencies.

Implementation of this PCR-based gene-disruption technique will preclude the generation of mutant strains from being the rate-limiting step in performing genetic analyses. The recently reported RNAi and antisense methods for disrupting gene function and the ability to generate panels of strains containing randomly inserted signature tags should also aid in accelerating the analysis of gene function (Gorlach *et al.*, 2002; Liu *et al.*, 2002; Nelson *et al.*, 2001). However, the PCR-based method proposed here has the potential to be applied to situations in which the ability to alter targeted genomic sequence is necessary. For instance, larger deletions that span multiple genes can be performed using this method. In addition to disruption mutations, this technique can also be applied to the efficient generation of other targeted insertions, including site-directed mutations and the insertion of regulatable promoters or epitope tags.

In conclusion, we show that this PCR-based gene-disruption approach is generally applicable for different genes using a variety of strains and genetic markers, and this should allow more efficient analysis of gene function in cases where the complete sequence is available.

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