Development of Positive Selectable Markers for the Fungal Pathogen *Cryptococcus neoformans*

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***Cryptococcus neoformans* is an opportunistic fungal pathogen that causes meningitis in ~10% of patients with AIDS. New selectable markers which confer resistance to G418 or phleomycin when transformed into *C. neoformans* were made. A hygromycin-selectable marker was modified to allow selection with a single copy of the marker.**

Cryptococcus neoformans is a haploid heterothallic basidiomycetous yeast that causes cryptococcal meningitis in immunocompromised patients, particularly those with AIDS (for a review, see reference 1). There are several different serotypes of *C. neoformans*, with serotype A being the most prevalent clinical isolate in the United States, followed by serotype D. *C. neoformans* is amenable to molecular genetics, with transformation systems and reverse genetics available (3–5). Although there are a few selectable markers available, most require the generation of a particular mutation in the recipient strain, including *ade2* (5), *ura5* (3), and *nmt487D* (4). A marker encoding resistance to hygromycin (2), which has been shown to be useful for transforming both wild-type strains and clinical isolates, was developed. Some studies, such as generation of insertion mutants and subsequent complementation or transformation with separate expression plasmids, require the use of multiple selectable markers. We have determined that *C. neoformans* is sensitive to several different antibiotics, including phleomycin and G418, and have constructed genes which encode resistance to phleomycin and G418, which can be used as selectable markers.

Determination of sensitivity of *C. neoformans* to antibiotics. Fresh colonies of the commonly used serotype A strain H99 were streaked onto YPD plates (1% yeast extract, 2% peptone, 2% glucose, 2% agar) plates containing the following antibiotics: phleomycin (Cayla) at 100, 10, or 1 µg/ml; G418 (Geneticin; Life Technologies) at 100, 10, or 1 µg/ml; netropsin (Roche) at 100 or 10 µg/ml; and oligomycin (Calbiochem) at 10, 1, or 0.1 µg/ml. *C. neoformans* showed good sensitivity in the assay to 100 µg of G418 per ml and 100 µg of netropsin per ml and some sensitivity to phleomycin at 100 µg/ml. No sensitivity to oligomycin was detected at any of the tested concentrations.

Phleomycin and G418 were tested further because there were genes available which confer resistance to these antibiotics in other systems. H99 cells were grown to late log phase, washed in phosphate-buffered saline (PBS), and plated at a concentration of ~10^7 cells per plate onto YPD agar plates containing different concentrations of antibiotic. It was determined that no detectable g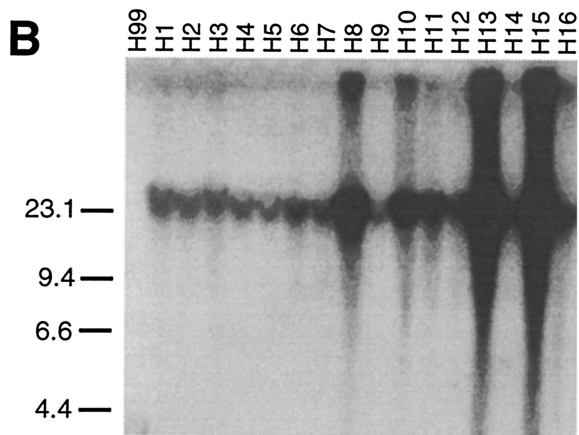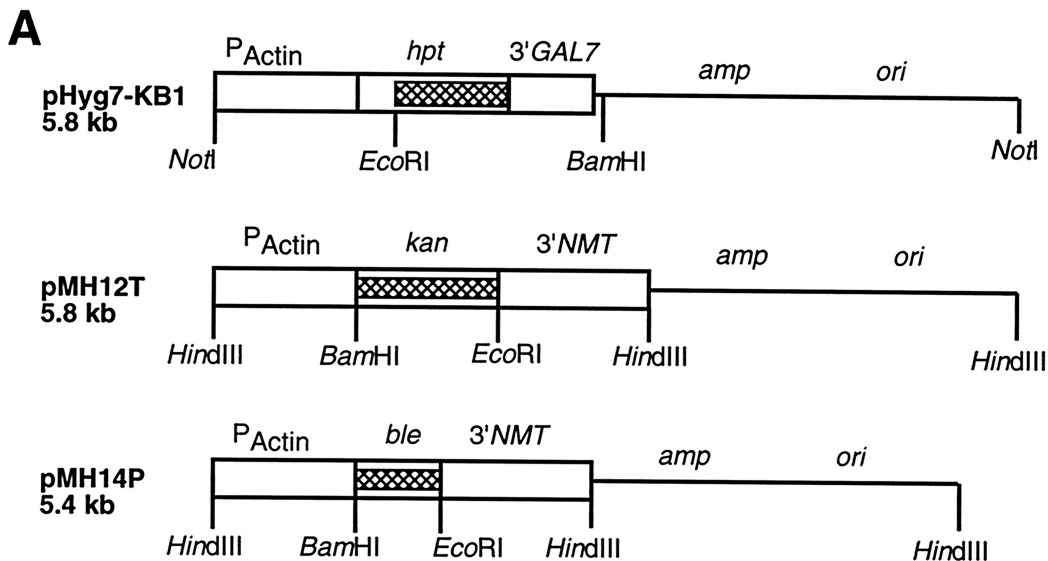rowth occurred on plates containing 200 µg of G418 per ml or on plates containing 250 µg of phleomycin per ml at pH 7.0.

Development of vectors for transformation studies. Figure 1A is a schematic of the three transformation vectors. To create the modified hygromycin resistance cassette, the plasmid pTELHYG (2) was digested with *Not*I and religated to remove the telomeric sequences and create plasmid pHYG. To provide a poly(A) addition site and possibly improve mRNA stability, the 3′ untranslated sequences from the *GAL7* gene (7) on plasmid pGUST11 (a generous gift from Brian Wickes) from the *Nru*I-*Kpn*I sites were inserted into the *Eco*RV-*Kpn*I sites just 3′ to the end of the hygromycin coding sequences (*hpt*) in the pHYG plasmid. The *Kpn*I site was then blunted, and a *Bam*HI linker was added to create the plasmid pHYG7-KB1.
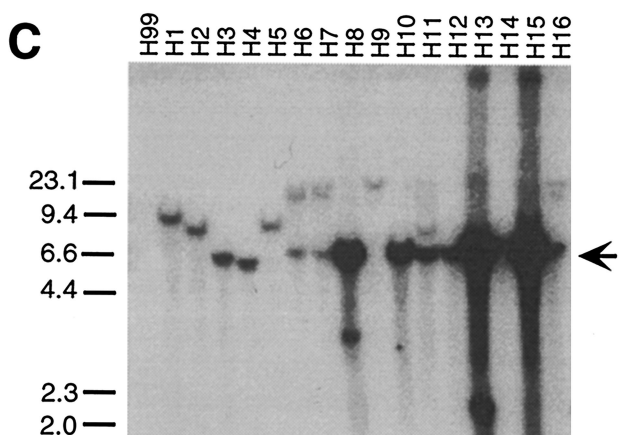
Coding sequences from the *kan* and *ble* genes from the bacterial transposable element Tn*5* were isolated by PCR with primers that introduced a *Bam*HI site just 5′ to the initiator ATG and an *Eco*RI site just 5′ to the terminator codon. To create pMH12-T, the synthesized 800-bp *kan* gene was cloned between an ~850-bp fragment containing the promoter from the *C. neoformans* actin gene (3) and an ~1,000-bp fragment containing the 3′ untranslated region from the *C. neoformans* NMT gene (4) in a pGEM13-derived vector. The synthesized ~450-bp *ble* gene was cloned into a similar vector to create pMH14-P. The PCR-synthesized *kan* and *ble* coding regions were sequenced, and no PCR-induced errors were incorporated into the genes. Similar plasmids containing the zeocin resistance coding sequence from pZERO (Invitrogen) and the *kan* gene from Tn*903* were also made and tested but never produced any transformants.

Transformation of *C. neoformans*. *C. neoformans* serotype A strain H99 and serotype D strain JEC20 were transformed by biolistic techniques (5). Plasmid DNAs used for the transformations were either uncut or cleaved with a restriction enzyme which cleaves once in the plasmid (see Fig. 1 and Table 1). The cells were grown to late log phase in YPD, concentrated, and plated onto YPD agar for transformation. The cells were bombarded with 0.6-µm-diameter gold particles coated with plasmid DNA and allowed to sit for 4 h on nonselective medium following transformation. The cells were resuspended in 0.5 ml of 1× PBS liquid medium and plated onto selective medium (YPD supplemented with 400 U of hygromycin (Calbiochem) per ml, YPD supplemented with 200 µg of G418 or YPD per ml, 10 mM MOPS (morpholine propanesulfonic acid; pH 7.0) supplemented with 250 µg of phleomycin per ml). Phleomycin
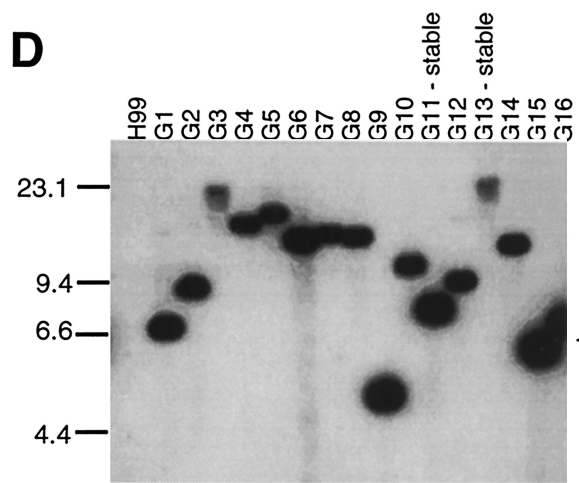
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A

pHyg7-KB1
5.8 kb

P_Actin   hpt   3'GAL7   amp   ori

NotI   EcoRI   BamHI   NotI

pMH12T
5.8 kb

P_Actin   kan   3'NMT   amp   ori

HindIII   BamHI   EcoRI   HindIII   HindIII

pMH14P
5.4 kb

P_Actin   ble   3'NMT   amp   ori

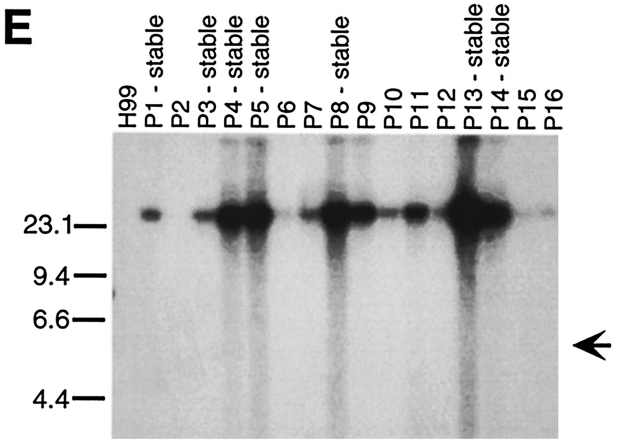HindIII   BamHI   EcoRI   HindIII   HindIII

B

Unrestricted - probed with hpt gene

C

Restricted with EcoRI - probed with hpt gene

D

Unrestricted - probed with kan gene

E

Unrestricted - probed with ble gene

TABLE 1. Transformation frequencies

Plasmid	Restricted[a]	Selection	Recipient strain	Frequency of transformants[b]	% Stable transformants[c]
pHYG7-KB1		Hygromycin	H99	20 ($\pm$16)	90
pHYG7-KB1	*Bam*HI	Hygromycin	H99	5 ($\pm$2)	83
pHYG7-KB1		Hygromycin	JEC20	36 ($\pm$34)	2
pMH12T		G418	H99	404 ($\pm$340)	6
pMH12T	*Not*I	G418	H99	180 ($\pm$158)	7
pMH12T		G418	JEC20	355 ($\pm$286)	8
pMH14P		Phleomycin	H99	125 ($\pm$169)	11
pMH14P	*Not*I	Phleomycin	H99	70 ($\pm$89)	5
pMH14P		Phleomycin	JEC20	0 ($\pm$0)	n/a

[a] The plasmids used for transformation were either uncut or restricted with the indicated restriction enzyme.

[b] The transformation frequencies are shown in transformants per microgram of DNA. They were determined from a minimum of six independent transformation events during at least two separate experiments. The standard deviation is shown in parenthesis.

[c] At least 50 independent transformants were passaged three times on nonselective medium and then tested for resistance on selective medium. n/a, not applicable.

plates were incubated at 4°C for 12 h before transfer to incubation at 30°C. All other plates were immediately incubated at 30°C. Transformants were observed within 3 to 7 days. The transformation frequencies for each condition, plasmid, and strain are shown in Table 1 and are given in transformants per microgram of plasmid DNA. Transformation with pMH12T was very efficient, with frequencies of well over 100/μg in both H99 and JEC20. Transformation with pHYG7-KB1 and pMH14P produced more modest frequencies, and we were unable to obtain any transformations with pMH14P with JEC20 as the recipient strain. The large standard deviations in the transformation frequencies reflects the variability of the process. We observed that different plates transformed at the same time with the same DNA preparation and cell culture would vary in transformation frequency and that there was even more variability from day to day.

Analysis of transformants. To determine whether the transformants were stable or unstable, independent transformants were passaged three times on nonselective YPD medium and then tested for resistance to the appropriate antibiotic; the results are shown in Table 1. Only those transformants that grew equally well on the selective medium as on nonselective medium were counted as stable transformants. We tested the stability of the transformants by passage in both liquid and solid medium and found no difference (data not shown). In H99, most of the hygromycin-resistant transformants were stable, whereas very few of the G418- or phleomycin-resistant transformants were stable. The percentage of stable transformants was unaffected by prior cleavage with a restriction enzyme. In JEC20, both the hygromycin- and G418-resistant transformants had a very low percentage of stable transformants.

In *C. neoformans*, unstable transformants are often the result of the transforming DNA forming extrachromosomal plasmids rather than integrating into the genome (3). These plasmids can often be detected by Southern blot analysis of uncut genomic DNA because they migrate significantly faster than the genomic DNA during agarose gel electrophoresis. Genomic DNA was isolated from cells grown on selective medium before they were passaged on nonselective medium. Unrestricted DNA or DNA cleaved with *Eco*RI was electrophoresed on 1% agarose gels and transferred to nylon membranes. The probes were fragments of the resistance genes, as indicated in Fig. 1A.

Southern blot analysis of the genomic DNA from 16 hygromycin-resistant H99 transformants using unrestricted genomic DNA (Figure 1B) and probed with a portion of the *hpt* gene showed that the hygromycin gene migrates with the genomic DNA and is not present as small extrachromosomal plasmids. All of these transformants were stable transformants. Southern blot analysis using the same genomic DNAs cleaved with *Eco*RI (Fig. 1C) indicated that several of the transformants (e.g., H1, H2, H3, H4, H5, and H9) had the marker present in a single copy integrated into the genome, as indicated by the presence of a single band. This result is in contrast to results with the parent plasmid, pTELHYG (2), where multiple insertion sites and extrachromosomal plasmids were observed in most transformants. The remaining transformants had multiple copies of the plasmid inserted in tandem arrays, as indicated by the presence of a band at 5.6 kb, the same size as pHYG7-KB1, and a second band indicating the flanking sequence. The flanking sequences are of different sizes, suggesting that the plasmid is inserted at different sites in the genome. These data suggest that pHYG7-KB1 would be a useful plasmid for experiments that require random integration into the genome.

Southern blot analysis of uncut genomic DNA from G418-resistant transformants (Fig. 1D) probed with the *kan* gene suggests that the transforming DNA is present as extrachromosomal plasmids and is not integrated into the genomic DNA. There was not a perfect correlation between the presence of plasmid DNA that migrates with the uncut genomic DNA and the stability of the transformant. For example, G11 was stable after multiple passages on nonselective medium, but the transforming DNA was clearly not integrated into the genome.

Southern blot analysis of the phleomycin-resistant transformants (Fig. 1E) showed that the *ble* gene probe hybridized to DNA that comigrated with the genomic DNA in most of the samples. Some samples showed little or no hybridization, but no samples showed hybridization to bands that migrated more quickly than the genomic DNA. However, more than half of the transformants tested were unstable. If the transforming

FIG. 1. (A) Schematic linear diagrams of the transformation plasmids. Relevant restriction sites are marked below the diagram. The portion of the plasmid used as a probe in the Southern blots are indicated with hatched boxes. (B, C, D, and E) Southern blot analysis of the transformants. H99 is the parental strain. H1 to H16 are stable hygromycin-resistant H99 transformants, G1 to G16 are G418-resistant H99 transformants, and P1 to P16 are phleomycin-resistant H99 transformants. Some of the G418 and phleomycin resistant transformants were stable, and those are marked above the lane. The size markers are indicated along the left side of the blots. Whether the genomic DNA is uncut or restricted with *Eco*RI is indicated below each blot. Uncut genomic DNA migrates with the 23.1-kb size marker. The position at which the linearized parent plasmid migrates is marked on the right of each blot with an arrow.

DNA is extrachromosomal, then it might be present as very large multimers of the plasmid or as an unstable minichromosome (6). Alternatively, the plasmid may be integrated into the chromosome in a manner that allows it to be lost very rapidly or it may be integrated into the mitochondrial genome. In general, the stable transformants had more signal than the unstable transformants, suggesting that the plasmid DNA was already being lost from the population of cells. Southern blot analysis using *Eco*RI-digested genomic DNA showed that the stable transformants had very high copy numbers of tandem arrays of the plasmid integrated into a single site (data not shown).

We have developed three (two new and one modified) positive selectable markers for use in transformation of the pathogenic fungus *C. neoformans*. The markers differ in transformation efficiency and frequency of stable integration into the genome. One possible source of the differences in transformation frequencies and the stability of the transformants is the difference in the 3′ sequences that were used. pHYG7-KB1 has a 3′ poly(A) site from the *GAL7* gene and produced a high percentage of stable transformants, whereas both pMH12T and pMH14P plasmids produced low percentages of stable transformants and have the 3′ poly(A) site from the *NMT* gene. The three plasmids clearly have different fates during transformation and therefore will have different utilities. The pHYG7-KB1 plasmid may be useful for generating stable insertions for insertional mutagenesis experiments. The pMH12T plasmid will be more useful for situations which require nonintegrative transformation. An example would be studies which require the transient expression of a protein, such as expression of an enzyme which modifies the genome

like transposase or Cre recombinase. In addition, this plasmid may be useful in complementation studies. The pMH14P plasmid may be most appropriate for developing stable transformants in which a very high copy number is desired, e.g., overexpression studies.

REFERENCES

1. **Casadevall, A., and J. R. Perfect.** 1998. *Cryptococcus neoformans*. ASM Press, Washington, D.C.
2. **Cox, G. M., D. L. Toffaletti, and J. R. Perfect.** 1996. Dominant selection system for use in *Cryptococcus neoformans*. J. Med. Vet. Mycol. **34:**385–391.
3. **Edman, J. C., and K. J. Kwon-Chung.** 1990. Isolation of the *URA5* gene from *Cryptococcus neoformans* var. *neoformans* and its use as a selective marker for transformation. Mol. Cell. Biol. **10:**4538–4544.
4. **Lodge, J. K., E. Jackson-Machelski, M. Higgins, B. Devadas, C. A. Mc-Wherter, and J. I. Gordon.** 1998. Genetic and biochemical studies establish that the fungicidal effect of a fully depeptidized inhibitor of *Cryptococcus neoformans* myristoylCoA:protein N-myristoyltransferase is Nmt dependent. J. Biol. Chem. **273:**12482–12491.
5. **Toffaletti, D. L., T. H. Rude, S. A. Johnston, D. T. Durack, and J. R. Perfect.** 1993. Gene transfer in *Cryptococcus neoformans* by use of biolistic delivery of DNA. J. Bacteriol. **175:**1405–1411.
6. **Varma, A., and K. J. Kwon-Chung.** 1994. Formation of a minichromosome in *Cryptococcus neoformans* as a result of electroporative transformation. Curr. Genet. **26:**54–61.
7. **Wickes, B. L., and J. C. Edman.** 1995. The *Cryptococcus neoformans GAL7* gene and its use as an inducible promoter. Mol. Microbiol. **16:**1099–1109.