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Dominant selection system for use in *Cryptococcus neoformans*

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Present transformation systems for *Cryptococcus neoformans* depend on complementation of auxotrophic mutants. We have developed a dominant selection system for transformation of wild-type strains of cryptococci in which resistance to the antibiotic hygromycin B is used as the selectable marker. A heterologous fusion gene construct was created by attaching the putative promoter sequence and start site from a cryptococcal actin gene to a truncated hygromycin B phosphotransferase gene from *E. coli*. Biolistic transformation with this construct resulted in cryptococci resistant to hygromycin B, and transformation efficiencies approached approximately 500 transformants per μg DNA. The construct was found to exist in transformants as both extrachromosomal and integrative forms. The transformants with integrated constructs were stable both *in vitro* and *in vivo*, and constructs were recoverable from most transformed cells using a plasmid rescue technique. This is the first dominant selection system for use in *C. neoformans*, and it should prove useful for molecular studies with this important pathogenic yeast.

Keywords cryptococcus, fungus, hygromycin B, transformation

Introduction

It has been more than 100 years since *Cryptococcus neoformans* was first implicated as a pathogen by Busse [1]. The number of infections caused by this encapsulated yeast have risen dramatically in recent years mostly as a result of the acquired immunodeficiency syndrome (AIDS) pandemic. The principal clinical manifestation of cryptococcal infection is meningitis, and this infection is fatal if untreated. Current treatment regimens for cryptococcal meningitis are associated with high toxicity and poor success rates. Therefore, research on the molecular pathogenesis of *C. neoformans* should yield important insights that may help identify unique targets for directed antifungal drug development.

There has been some progress in the molecular understanding of *C. neoformans*, but this fungal pathogen remains a genetically undeveloped organism. There are two transformation systems available; one uses electroporation [2] and the other uses biolistics [3] for DNA delivery. However, both systems depend on complementation of auxotrophic mutants. A transformation system using a dominant selection marker would be particularly advantageous as wild-type cryptococci could be studied, and it would obviate the use of auxotrophic mutants which may have other unspecified mutations. In this study we describe the development of a dominant selection system for use in wild-type *C. neoformans*.

We chose expression of the hygromycin B phosphotransferase gene (*HYGB*) from *Escherichia coli* as the selectable marker. This gene encodes for an enzyme that inactivates the aminocyclitol antibiotic hygromycin B thus conferring resistance, and has been used in several other fungal transformation systems where it has been fused to a variety of eukaryotic promoter sequences [4–9]. We tested well over 10^9 yeasts of the *C. neoformans* strain H99 and did not find a spontaneously hygromycin B-resistant isolate when the yeast was grown on yeast extract, peptone, dextrose (YEPD) medium containing hygromycin B at levels of 200 U ml^{-1}. We therefore reasoned that resistance to this drug would be an excellent selectable marker for this yeast if the *E. coli* hygromycin B phosphotransferase gene could be delivered and expressed in *C. neoformans*.

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Materials and methods

Strains

The *C. neoformans* strains used in these experiments were H99 (serotype A, mat *a*), N32 (serotype B, mat *a*), N34 (serotype C, mat *a*), 3501 (serotype D, mat *a*), and three clinical isolates (two were obtained from the cerebrospinal fluid from patients with cryptococcal meningitis and AIDS and one was from a lung biopsy specimen of a patient previously treated with corticosteroids). The clinical isolates were identified using standard NCCLS criteria, and were not serotyped. Strain 3501 was provided by Dr K. J. Kwon-Chung (National Institutes of Health, Bethesda, MD), and strains N32 and N34 were provided by Dr Michael McGinnis (University of Texas—Health Sciences Branch, Galveston, TX).

Construction of vectors

A copy of the *E. coli* hygromycin B phosphotransferase gene (*HYGB*) contained in the plasmid p213 (kindly provided by T. D. Ingolia, Eli Lilly Pharmaceuticals [6]), was cut out by digestion with *Bam*HI and *Hin*dIII and subcloned into pBluescript SK− (Stratagene). This plasmid was amplified and the truncated *HYGB* was cut out by restricting with *Bam*HI and *Apa*I. Dr Jeff Edman graciously provided us with a copy of the pCnTEL plasmid which contains telomere-like sequences to provide stability in transformed cryptococci [2]. We modified this plasmid by restricting with *Bam*HI and *Apa*I to cut out *URA5*, and then subcloned the truncated *HYGB* into the modified pCnTEL. An 862 bp fragment from the *C. neoformans* strain H99 actin gene [10] extending from 847 bp upstream of the putative ATG start site to 15 bp downstream was isolated by restricting the cloned genomic fragment containing the actin gene with *Bam*HI and *Eco*RV to result in a 2·5 kb fragment. This fragment was further digested with *Rsa*I and the 862 bp fragment was identified using electrophoresis and isolated using electroelution. The modified pCnTEL plasmid containing *HYGB* was digested with *Bam*HI (which cuts at the site of the start of the truncated *HYGB*) and a fill-in reaction was performed to result in blunt ends. The 862 bp actin fragment was then ligated in-frame to the 5′ end of *HYGB*, and the correct orientation of the fusion was confirmed by digestion with *Sty*I. This construct was labelled pTelHyg (Fig. 1).

Transformation procedure

Plasmid preparations of pTelHyg were amplified in *E. coli* strain HB101 grown in LB broth under selective conditions of ampicillin 50 μg ml^{-1} and kanamycin 35 μg ml^{-1}. Plasmids were isolated and linearized by digestion with *Sce*I (Boehringer-Mannheim). The plasmids were then extracted once with 1:1 phenol:chloroform, precipitated with 95% ethanol, and brought up in TE (pH 8·0). Transformation of *C. neoformans* strain H99 with both pTelHyg as well as control plasmid containing a promoter-less copy of *HYGB* was carried out using biolistics as described previously [3] with the exception that each transformation event was performed with approximately 0·5 μg linearized plasmid. The yeast were initially transformed on YEPD medium, and then 1 ml of regeneration medium was added [3] to each plate immediately after transformation. The plates containing the regeneration medium were rocked for 2 h and the supernatants were collected and divided into two equal portions and plated onto YEPD plates containing 200 units hygromycin B/ml (LC Pharmaceuticals, Woburn, MA) and incubated at 30 °C for 5 days. Transformation using electroporation was carried out as described by Edman [2]. The actual charging voltage was 490 with a capacitance of 50 μF, resistance of 360 Ω and a time constant ranging from 18 to 22 ms.

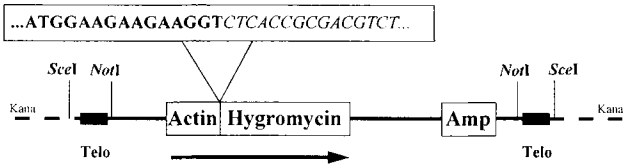

Fig. 1 The pTelHyg construct. *C. neoformans* actin promoter (Actin) was fused to an *E. coli* hygromycin B phosphotransferase gene (Hygromycin). The junction of the actin promoter and hygromycin B phosphotransferase gene is shown in the box above the fusion. The first 15 base pairs in bold are from the actin gene and begin with the ATG start site. The next 15 base pairs in italics are from the 5′ end of the hygromycin B phosphotransferase gene. When digested with *Sce*I, the kanamycin resistance gene (*Kana*) is cut out and the linearized plasmid is flanked by telomere-like sequences (Telo). Recovery of the plasmid can be accomplished by restricting with *Not*I to cut off the telomere-like sequences and then ligating the plasmid to itself. Amp, ampicillin resistance gene; *Not*I, *Not*I restriction enzyme site; *Sce*I, *Sce*I restriction enzyme site.

DNA analysis

Isolation of DNA from the transformed yeast was carried out using a glass bead method as described by Fujimura & Sakuma [11] using yeasts harvested from YEPD plates containing 200 units hygromycin B/ml. Karyotype gels were prepared as described previously [12]. Southern analysis of transformant DNA preparation was carried out according to Sambrook *et al.* [13]. Probes were made using a random primer kit (Gibco-BRL) and ^{32}P-dATP (New England Nuclear) using *HYGB* as template.

Autoradiographs were exposed overnight at −70 °C on Kodak XAR film.

In vivo *testing*

The pTelHyg plasmid was tested for *in vivo* stability by using the rabbit cryptococcal meningitis model [14]. A total of 10^6 hygromycin B-resistant yeast of a single transformant were inoculated into the cisterna magna of four rabbits. One additional rabbit was inoculated with an equal number of H99. Serial taps of the cerebrospinal fluid (CSF) were performed on days 1, 4, 7 and 10 after inoculation. The CSF samples were plated for quantitative counts on both YEPD medium and YEPD medium containing 200 units hygromycin B/ml. Colony counts were made after incubation for 3 days at 30 °C.

Plasmid rescue

DNA preparations of the transformed yeast were digested with *Not*I and then extracted × 2 with 1:1 phenol: chloroform. The DNA was precipitated with 95% ethanol and then brought up in 1 x ligase buffer with 0·01 units ligase (Gibco-BRL) per μg DNA. After ligation overnight at 4 °C, the DNA was used to transform $CaCl_2$-treated competent *E. coli* strains TG1 or HB101. The transformants were selected on LB plates with 50 μg ampicillin/ml.

Results

A heterologous fusion gene was made using the putative promoter and start site sequences from a *C. neoformans* actin gene ligated in frame to a truncated copy of the *E. coli* hygromycin B phosphotransferase gene. This fusion gene was inserted into a modified pCnTEL plasmid [2] resulting in pTelHyg (Fig. 1). When linearized with *Sce*I digestion, this construct has telomere-like sequences flanking the remaining plasmid DNA to confer stability after transformation. Biolistic transformation of cryptococci with pTelHyg resulted in yeasts resistant to hygromycin B. There were approximately 200–250 resistant colonies per transformation event using 0·5 μg of linearized plasmid DNA when strain H99 was transformed (Table 1). We were also able to transform cryptococcal strains recently taken from clinical specimens as well as representative *C. neoformans* strains of serotypes B, C and D to hygromycin B resistance using this construct. However, the relative efficiencies for these other strains were significantly lower than for H99. Only a single resistant colony was obtained after three transformation events for each of the serotype B, C and D strains. Transformation of strain H99 with pTelHyg was also accomplished by electroporation although with much lower efficiency.

Table 1 Results of transformation

Strain	No. of resistant colonies Average of 3 experiments (range)
H99	227 (120–380)
Clin1	12 (1–29)
Clin2	18 (2–40)
Clin3	28 (14–46)
H99(electroporation)	53 (35–65)

Different cryptococcal strains were transformed with pTelHyg and the resulting numbers of hygromycin B-resistant colonies are shown. Biolistic transformation was used for all experiments unless specified. The data listed for strain H99 used 0·5 μg of plasmid DNA per transformation event while 0·25 μg of DNA was used for the others. Clin1, Clin2 and Clin3 were obtained from patients with cryptococcal infections and were not serotyped.

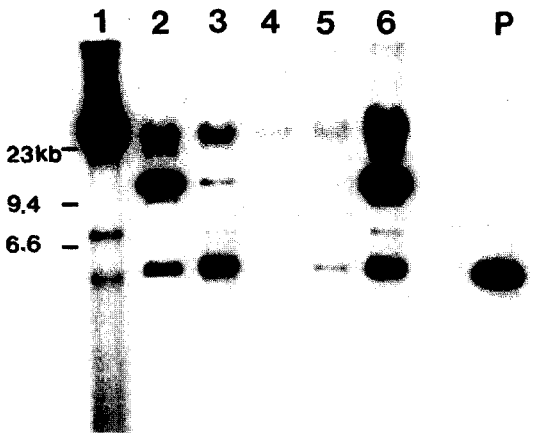

Fig. 2 Autoradiograph of a Southern blot hybridized with the *HYGB* probe. Undigested genomic DNA prepared from six different transformants is in lane nos 1–6. Lane P contains pTelHyg digested with *Not*I. Molecular weight standards in kilobases are given on the left.

When electroporation was used to deliver the DNA, we were only able to achieve approximately one-fourth the number of transformants per μg DNA that were obtained using the biolistic system (Table 1).

Southern analysis of six H99 transformants showed that the construct existed in two forms. Each DNA preparation showed hybridization to the *HYGB* probe at a 5·65 kb band that corresponds to the molecular weight of the extrachromosomal plasmid construct, and each had hybridization to higher molecular weight bands corresponding to genomic DNA, dimers or other extrachromosomal multimeric repeats (Fig. 2). The same hybridization patterns were seen in the transformants from two of the three clinical isolates and the serotype B strain, but one of the clinical isolates and the serotype C

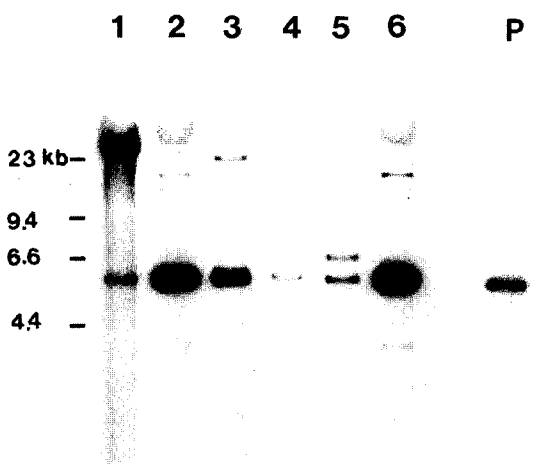

Fig. 3 Autoradiograph of a Southern blot probed with the *HYGB* probe. Lanes 1–6 each contain genomic DNA from the same transformants shown in Fig. 2 that has been digested with *Not*I. Lane P contains pTelHyg digested with *Not*I. Molecular weight standards in kilobases are given on the left.

and D strains demonstrated hybridization to the extrachromosomal forms only (data not shown). Southern analysis of the same 6 H99 transformant DNA preparations from Fig. 2 digested with *Not*I and probed with *HYGB* showed that all had hybridization at the expected 5·5 kb band (corresponding to the size of the construct minus the telomere-like sequences), and most of the higher molecular weight bands were diminished in intensity suggesting that some of the high molecular weight signal consisted of multimeric repeats or genomic inserts that were digested to the 5·5 kb size (Fig. 3). However, there were still signals found at the high molecular weight bands and it was thought that these represented integration into the genome with loss of the flanking *Not*I sites. In order to confirm this, Southern analysis of karyotypes from the same 6 H99 transformants used in the previous figures in shown in Fig. 4. The karyotype blot shows that there are a series of different patterns of integration into the chromosomes for each of the transformants studied. Most of the transformants tested showed hybridization to multiple chromosomes, and the karyotypes of five transformants (nos 2–6) showed the development of different-sized chromosomes. Transformants nos 2, 3 and 4 each showed a new chromosome band between the 1·6 and 1·7 mb bands; transformants nos 4 and 5 each showed a new chromosome band between the 1·7 and 2·0 mb bands; transformant no. 6 showed a new band just above the 1·13 mb chromosome band. Transformant no. 5 actually showed decreased signal from the expected position of the 1·13 mb chromosome band. Of the six new chromosome bands that were seen in the transformants, four demonstrated hybridization to the *HYGB* probe. The new chromosome bands between the 1·7 and 2·0 mb bands for transformants nos 4 and 5 were the only new bands that did not show hybridization.

Cryptococci transformed with pTelHyg were stable after multiple passages on selective medium containing hygromycin B concentrations of 200 units ml^{-1}. However, when the transformants were passed on non-selective medium, the numbers of hygromycin B-sensitive colonies increased significantly. Because of the apparent loss of the construct under non-selective conditions, there was a concern that the construct would not be stable *in vivo*. Therefore, rabbits were infected with a randomly chosen H99 transformant and the CSF was sampled and plated onto both selective and non-selective media for colony counts. Table 2 shows both the total numbers of cryptococci and the numbers of hygromycin B-resistant cryptococci that were recovered during serial CSF withdrawals from the infected rabbits. The percentage of hygromycin B-resistant cryptococci of the total population in the CSF decreased over time. Preparations of DNA from hygromycin B-resistant yeast recovered from the CSF showed hybridization of high molecular weight DNA to the *HYGB* probe (data not shown). However, only one resistant isolate out of 12 tested showed hybridization to the 5·65 kb band, corresponding to the extrachromosomal construct, and it was obtained on day 1 after inoculation. This demonstrates the rapid loss of the episomal form when transformants are recovered from the site of infection.

The pTelHyg construct was recoverable from transformed *C. neoformans* using a plasmid rescue technique [2]. Whole DNA preparations of the transformants were digested with *Not*I to remove the telomere-like sequences from the construct and were then self-ligated. This DNA mixture was used to transform *E. coli* and transformants were selected on plates containing ampicillin. We were able to recover the plasmid construct from four of six transformants. The two from which we could not recover the plasmid using this technique had no extrachromosomal forms on Southern analysis, and we assume that the construct had integrated into the genome with loss of the flanking *Not*I sites. The integrated construct that has lost the flanking *Not*I sites may be recoverable using other techniques such as the polymerase chain reaction (PCR).

Discussion

This paper describes the creation of a novel transformation system for use in *C. neoformans*. Resistance to hygromycin B was used as a dominant selection marker, and as opposed to the other existing transformation systems used with *C. neoformans*, this system is not

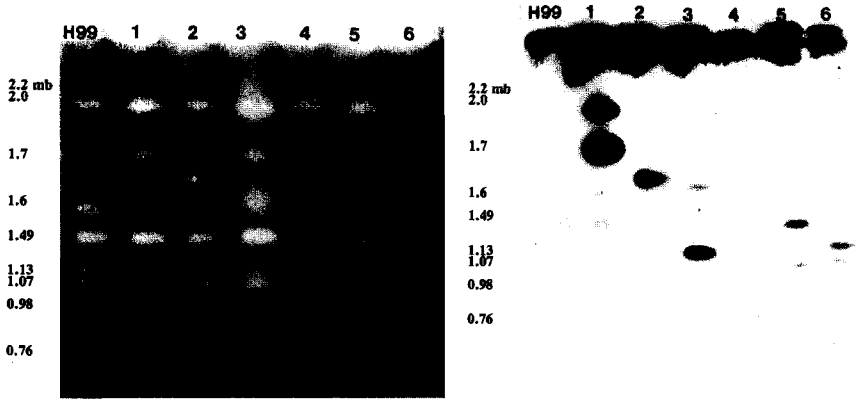

Fig. 4 Karyotype gel (left) and its Southern blot (right) of H99 and the six transformants from Figs 2 and 3. Molecular weight standards in megabases are given on the left. Karyotyping was carried out as described by Perfect *et al.* [12]. The Southern blot was probed with the *HYGB* probe. Five of the six transformants show the appearance of new chromosome bands and four of the six show hybridization to multiple chromosomes (see Results).

Table 2 Stability of transformants *in vivo*

	No. of hygromycin B-resistant colonies/total recovered (%)			
Infecting strain	Day 1	Day 4	Day 7	Day 10
Transformant	1370/1607 (85)	626/844 (74)	145/162 (90)	143/246 (58)
H99 (wild-type)	0/386 (0)	0/234 (0)	0/66 (0)	0/59 (0)

Rabbits were experimentally infected with either hygromycin B-resistant yeast transformed with the pTelHyg construct (4 rabbits) or an H99 control (1 rabbit). The CSF was serially sampled and plated on both selective and non-selective media. The numbers represent the total number of colonies counted after plating 0·1 ml of CSF.

limited for use in auxotrophic strains. This transformation system was specifically designed for use in strain H99 and other serotype A strains. The extremely low rates of transformation for the serotype B, C and D strains may be accounted for by the fact that the promoter sequence is from a serotype A strain (H99). It is possible that the promoter will not be efficiently recognized by other strains especially when they are of B, C or D serotypes. This supposition is based on observations of extensive polymorphisms between cryptococcal strains found in at least two genes. We have found that the *ADE2* genes of various serotypes contain significant nucleotide variation in the coding regions and widely divergent sequences in the noncoding regions (unpublished data). Casadevall *et al.* compared the *URA5* sequences of different cryptococcal strains and found nucleotide sequence variations of approximately 5–7% [15]. There are not yet enough sequence data to identify promoter motifs for *C. neoformans*, but given the wide variation in DNA sequences between strains that we know of so far it seems reasonable to assume that promoter regions can be relatively serotype-specific.

The construct used in these experiments contained a heterologous fusion gene composed of a cryptococcal promoter fused to a procaryotic resistance gene. We have also found that β-galactosidase gene fusions can be expressed in strain H99 (unpublished data) and Wickes & Edman have been able to express β-glucoronidase in another *C. neoformans* strain [16]. These functional fusion genes demonstrate that some bacterial genes can be expressed in *C. neoformans*. This is in contrast to the pathogenic fungus *Candida albicans* which cannot express procaryotic genes because of codon usage differences.

The transformation efficiency of 454 per μg found in this study using biolistic transformation is much lower than efficiencies of approximately 10^4 found by Edman using pCnTEL and electroporation [2]. There are many variables to consider to account for this difference, among which the most important are the method of transformation and the strain of cryptococci used. We were able to transform strain H99 with pTelHyg using electroporation, but at lower efficiencies than with biolistics. It is possible that we could increase the efficiency by rigorously optimizing the electroporation conditions. It is also possible that strain H99 is not as amenable to electroporation as are the serotype D strains used by Edman.

Transformation with pTelHyg resulted in both extrachromosomal and integrated constructs for all H99 transformants tested. The extrachromosomal forms were stable during passage under selective conditions, but were quickly lost by the yeast when the transformants were inoculated into the CSF of rabbits. Integration into the

genome with pTelHyg is likely random as the patterns on Southern analysis of both genomic digests and karyotypes are different for each of the transformants tested. The only cryptococcal DNA that the construct contains is from the actin gene and the telomere-like sequences. Disruption of the actin gene in *Saccharomyces* is lethal [17] therefore it is likely that homologous integration at this locus is not possible in *Cryptococcus* as it too contains a single actin gene [10]. It is possible that integration occurred at the telomere sequences of the cryptococcal chromosomes, and this report shows that vectors containing telomere-like sequences can still integrate within the genome. The rationale for using a modified pCnTEL as a vector in these experiments was that the telomere-like sequences on this plasmid would provide stability to an episomal construct after transformation. The original studies of pCnTEL did not document integrative events after transformation when electroporation was used [2]. However, using the same basic construct in these experiments with biolistic transformation we obtained integration in every transformant tested. Biolistic transformation may more often result in integration compared with electroporation, and it is also possible that strain H99 is more amenable to integration than other strains. An episomal construct that did not integrate may be desired in certain situations since integration can potentially result in unspecified mutations. It is clear that the multiple integrative events obtained after transformation with pTelHyg limit its utility for some applications. The multiple integrative events seen with this construct may be necessary if multiple copies of the resistance gene are needed to confer resistance in the yeasts. If this is the case, then efforts at increasing transcription of the resistance gene by using different promoters or adding transcription termination sequences may increase transformation efficiency and allow single copies of the gene to confer resistance. However, the actin-hygromycin resistance gene cassette can be used independent of the telomere-containing plasmid. We are currently investigating the use of this resistance cassette in studies such as restriction enzyme-mediated insertional mutagenesis, signature-tagged mutagenesis and gene disruption experiments. It will be helpful in these applications to optimize the gene cassette so it can confer resistance with a single copy, and efforts to investigate this are currently underway.

Transformation with pTelHyg resulted in the appearance of new chromosomes, most of which demonstrated hybridization to the *HYGB* probe. The appearance of a new chromosome as a result of transformation has been described previously for *C. neoformans*. Varma & Kwon-Chung found a minichromosome of approximately 270 kb size after electroporative transformation [18]. We found new chromosome bands in three of six transformants tested, and the sizes ranged from approximately 1·13 to 1·8 Mb. The new chromosomes containing the construct may have resulted from autoexcision of the integrated DNA along with a large portion of the original chromosome as postulated by Varma & Kwon-Chung [18]. However, the two new chromosome bands that did not hybridize to the *HYGB* probe may have resulted from the mechanical stress of biolistic transformation. It is also possible that the presence of the telomere-like sequences on the vector results in a higher frequency of chromosome rearrangement compared with vectors without these sequences. Although the mechanisms for these karyotypic variations remain uncertain, the results of these shifting chromosomes emphasize the impressive plasticity of some fungal chromosomes [19].

Acknowledgements

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