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Transformation of *Candida albicans* with a synthetic hygromycin B resistance gene

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Abstract

Synthetic genes that confer resistance to the antibiotic nourseothricin in the pathogenic fungus *Candida albicans* are available, but genes conferring resistance to other antibiotics are not. We found that multiple *C. albicans* strains were inhibited by hygromycin B, so we designed a 1026 bp gene (*CaHygB*) that encodes *Escherichia coli* hygromycin B phosphotransferase with *C. albicans* codons. *CaHygB* conferred hygromycin B resistance in *C. albicans* transformed with *ars2*-containing plasmids or single-copy integrating vectors. Since *CaHygB* did not confer nourseothricin resistance and since the nourseothricin resistance marker *SAT-1* did not confer hygromycin B resistance, we reasoned that these two markers could be used for homologous gene disruptions in wild-type *C. albicans*. We used PCR to fuse *CaHygB* or *SAT-1* to approximately 1 kb of 5' and 3' noncoding DNA from *C. albicans ARG4*, *HIS1* and *LEU2*, and we introduced the resulting amplicons into 6 wild-type *C. albicans* strains. Homologous targeting frequencies were approximately 50-70%, and disruption of both *ARG4*, *HIS1* and *LEU2* alleles was verified by the respective transformants' inabilities to grow without arginine, histidine and leucine. *CaHygB* should be a useful tool for genetic manipulation of different *C. albicans* strains, including clinical isolates.

INTRODUCTION

Candida albicans was the first medically-important fungus for which integrative and episomal DNA transformation systems were developed. A large number of *C. albicans* mutants have been constructed by homologous gene targeting, most of which were generated by transforming auxotrophic mutants with the corresponding nutritional markers. This approach has been extremely useful, but it also has limitations. For example, the *ura3* null mutation has phenotypic consequences other than nutritional auxotrophy, and these

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non-nutritional phenotypes are reversed to variable extents when *URA3* is reintegrated into different chromosomal sites (for review, Staab and Sundstrom 2003; Brand *et al.*, 2004). Also, nutritional markers cannot be used to study proposed virulence determinants in clinical isolates because these wild-type strains lack nutritional auxotrophies. For reasons such as these, there has been considerable interest in developing dominant selection markers that function in *C. albicans*. Two groups have shown that overexpression of *C. albicans IMH3* conferred resistance to mycophenolic acid in *C. albicans* transformants (Köhler *et al.*, 1997; Beckerman *et al.*, 2001), but this marker has not been used subsequently. More recently, three groups generated synthetic markers that conferred resistance to the antibiotic nourseothricin, and these markers have since been used to construct many *C. albicans* mutants (Roemer e*t al.*, 2003; Reuss *et al.*, 2004; Shen *et al.*, 2005). Since *C. albicans* has a diploid genome, two separate homologous gene targeting steps are required to create homozygous mutants. Reuss *et al.* (2004) addressed this problem by constructing a gene targeting cassette in which the nourseothricin resistance marker can be excised by activating an internal FLP recombinase. Since this marker can be recycled, it can be used to target both chromosomal alleles of a gene of interest. An alternative approach would be to use a second antibiotic resistance marker to target the second chromosomal allele of a gene of interest, but the antibiotic resistance markers that are commonly used in other organisms do not function in *C. albicans*.

The aminoglycoside antibiotic hygromycin B inhibits protein synthesis (Singh *et al.*, 1979), and hygromycin B resistance genes have been used in DNA transformation of many organisms (Kaster *et al.*, 1983; Shimamoto *et al.*, 1993; Giordano and McAllister, 1990). Genes derived from other hosts often do not function in *C. albicans* because the codon CTG encodes leucine in *C. albicans* and a few other *Candida* species, whereas CTG encodes serine in almost all other organisms (Pesole *et al.*, 1995). Hara *et al*. (2000) used site-directed mutagenesis to replace the 9 CTG codons in the *E. coli* hygromycin B phosphotransferase gene with alternative leucine codons and found that the resulting hygromycin B resistance gene (*HYG#*) conferred hygromycin B resistance in *Candida tropicalis*. In preliminary experiments, we transformed *C. albicans* with the *HYG#* gene under the control of the *PGK* promoter, but the resulting transformants did not grow on rich media containing 600 μg hygromycin B per ml (unpublished data). Since changing only the CTG codons to alternative leucine codons may not be sufficient to permit optimal expression of heterologous genes in *C. albicans* (Cormack *et al.*, 1997; Shen *et al.*, 2005), we designed and synthesized a hygromycin B phosphotransferase gene with optimized *C. albicans* codons. This report describes this synthetic gene's ability to confer hygromycin B resistance when introduced into *C. albicans* transformants in single and multiple copy vectors and also a new fusion PCR method that uses the synthetic hygromycin B gene to introduce null mutations into wild-type *C. albicans* strains.

MATERIALS AND METHODS

Strains and media

C. albicans strains SC5314 and its *ura3* derivative CAI4 were obtained from W. Fonzi (Georgetown Univ.), *C. albicans* WO-1 was from P.T. Magee (Univ. of Minnesota), *C.*

albicans B311 was from H. Buckley (Temple Univ.), and *C. albicans* strain YPT1 (Table1) (containing the nourseothricin resistance gene *SAT-1*) was from T. Roemer (Mycota Biosciences, Montreal, Canada). *C. albicans* strains CT98001-5001, CT98004-5004 and CT98009-5009 were bloodstream isolates from patients in a population-based study of *Candida* fungemias (Hajjeh *et al.*, 2004).

C. albicans was cultured in YP medium (yeast extract 1%; peptone 2%) containing glucose (2%) or maltose (2%); in minimal medium (YNB) (0.67% yeast nitrogen base without amino acids containing glucose (2%); or in buffered YNB (YNB buffered to pH 7.0 with 0.15 M Hepes-NaOH). The media listed above were supplemented with graded amounts (200-1200 μg per ml) of hygromycin B (A.G. scientific Inc, USA) and/or nourseothricin (Werner Bioagents, Germany) at 400 μg/ml.

Plasmids were maintained in *Escherichia coli DH5*α grown in Luria-Bertani medium (LB) containing 100 μg ampicillin per ml.

Design and synthesis of *CaHygB*

A synthetic hygromycin B resistance gene with optimized *C. albicans* codons (*CaHygB*) was designed by reverse transcription of the 342 amino acids in *E. coli* hygromycin B phosphotransferase (Gritz and Davies, 1983) with the most frequent codon encoding each amino acid in a *C. albicans* codon usage table (http://www.kazusa.or.jp/codon/cgi bin/showcodon.cgi?species=5476), except that the second most frequent codon was used in a few cases to remove inconvenient restriction endonuclease sites or to introduce convenient ones. The resulting 1026 bp *CaHygB* coding sequence flanked by an *Xho*I restriction site at the 5' end and a *Bam*HI site at the 3' end (Genbank accession number GU938191) was synthesized and ligated into plasmid pCRII (Invitrogen) by a commercial vendor (Bionexus Inc, Oakland, CA), and the accuracy of the DNA synthesis was verified by DNA sequencing.

Plasmid construction and transformation

Plasmid pBSII-CaHygB was constructed by inserting (i) the *C. albicans TEF2* promoter [amplified from *C. albicans* SC5314 genomic DNA by PCR with primers TEF2pt-5 and TEF2pt-3 (Table 1)] into the the *Kpn*I and *Xho*I sites of pBluescript II SK+ (Stratagene), (ii) the *CaHygB* marker into the resulting plasmid's *Xho*I and *Bam*HI sites, and (iii) the *C. albicans ACT1* terminator (amplified from *C. albicans* SC5314 genomic DNA by PCR with primers ACT1tm-5 and ACT1tm-3) into the *Bam*HI and *Xba*I sites of the resulting plasmid (Table 3). Plasmid pYM70 (Fig 1) was constructed by ligating into pUC18 (i) an *Nde*I fragment from pCaARS2 that contains *C. albicans ARS2* (Cannon *et al.*, 1990), (ii) the *Xho*I-*Bam*HI fragment from pBSII-CaHygB that contains *CaHygB* flanked by the *C. albicans TEF2* promoter and *ACT1* terminator, (iii) the *Sac*I-*Sac*II fragment from pYM6 that contain the *C. albicans TEF2* terminator (Mao *et al.*, 1999), and (iv) the *C. albicans ACT1* promoter (amplified from *C. albicans* SC5314 genomic DNA by PCR with primers ACT1pt-5 and ACT1pt-3) (Table 3). The DNA sequence of plasmid pYM70 has been deposited in Genbank (accession number GU937092). Plasmid pYM70 is available to academic researchers by writing to Brian Wong (wongbri@ohsu.edu).

Plasmids pAU34-CaHygB and pAU15-CaHygB were constructed by inserting the *CaHygB* marker into the *Xho*I and *Bam*HI sites in the *ACT1*-regulated integrating vector pAU34 and the *MAL2*-regulated integrating vector pAU15, respectively (Uhl and Johnson, 2001).

The nourseothricin-conferring *SAT-1* cassette consists of a synthetic *SAT-1* gene with optimal *C. albicans* codons flanked by the *C. albicans ACT1* promoter and the *PCK1* terminator (Roemer *et al.*, 2003).

Plasmids were introduced into *C. albicans* using the lithium acetate method (Walther and Wendland, 2003), and transformants were selected on minimal media lacking uridine or on YP or buffered YNB media containing hygromycin B or nourseothricin.

Strain construction by double fusion PCR

The double fusion PCR strategy (Amberg *et al.*, 1995) was adapted to replace one chromosomal *ARG4*, *HIS1* and *LEU2* allele in *C. albicans* with the *CaHygB* marker and the second allele of each gene with the *SAT-1* marker. Briefly, we used PCR to amplify (i) approximately 1 kb of *C. albicans* SC5314 genomic DNA from the 5' region flanking each ORF of interest, (ii) the *CaHygB* or the *SAT-1* marker, and (iii) approximately 1 kb of *C. albicans* SC5314 genomic DNA from the 3' region flanking the ORF. The oligonucleotides used for these PCRs (Table 3) were designed so that the 3' end of each 5' flanking region and the 5' end of each 3' flanking region were complementary to the 5' and 3' ends, respectively, of the *CaHygB* and *SAT-1* markers. Therefore, a PCR reaction that uses approximately equal amounts of the three gel-purified amplicons of interest as templates and primers complementary to the 5' terminus of the 5' flanking DNA and the 3' terminus of the 3' flanking DNA should generate a single fusion of the 5' flanking region, the selectable marker of interest, and the 3' flanking region. The adequacy of each final PCR reaction was assessed by agarose gel electrophoresis, and conditions were adjusted to maximize the yield of the desired products.

Once PCR conditions were optimized to yield the desired full-length amplicons, the *C. albicans* strains were transformed directly with the PCR products using the lithium acetate method (Walther and Wendland, 2003), and transformants were selected on the appropriate antibiotic-containing media.

Genomic DNA was amplified by PCR with the verification primers in Table 3 to determine (i) if the ORFs of interest were present or absent, (ii) the overall sizes of the chromosomal loci of interest, and (iii) if the *CaHygB* and/or the *SAT-1* markers had integrated homologously into the chromosomal loci of interest. Several verification primers derived from the 3' end of *ARG4* were required to generate diagnostic amplicons from different *C. albicans* strains.

Phenotypic analyses

C. albicans transformants were tested for drug resistance by testing for growth at 30°C on solid YP-glucose, YNB-glucose or buffered YNB-glucose containing hygromycin B and/or nourseothricin, and they were tested for nutritional auxotrophies on buffered YNB-glucose lacking histidine, arginine or leucine.

RESULTS

Susceptibility of *C. albicans* strains to hygromycin B and nourseothricin

C. albicans strains SC5314, CAI4, WO-1, B311, CT98001-5001, CT98004-5004 and CT98009-5009 did not grow in YP-glucose containing 600 μg hygromycin B per ml. All of these strains grew well in YNB-glucose containing 1200 μg hygromycin B per ml, but they did not grow in buffered YNB-glucose (pH 7.0, 0.15 M Hepes-NaOH) containing 1000 μg hygromycin B per ml. All of the *C. albicans* strains were inhibited by 400 μg nourseothricin per ml in YP-glucose or in buffered YNB-glucose.

Properties of *CaHygB*-containing plasmids

To determine if multicopy plasmids encoding the *CaHygB* marker would confer hygromycin B resistance, *C. albicans* CAI4 was transformed with plasmid pYM70. The resulting transformants grew well in YP-glucose + 600 μg hygromycin B per ml (Fig 2). We used two approaches to determine if pYM70 replicated in *C. albicans* as extrachromosomal episomes. First, the stability of pYM70 in the absence of hygromycin B selection was examined (i) by culturing 12 independent pYM70 transformants for 40 generations in liquid YP-glucose or in YP-glucose + hygromycin B and (ii) by plating serial dilutions of each cell suspension onto solid YP-glucose or YP-glucose + hygromycin B. After 40 generations in YP-glucose without hygromycin B, there were 0.71 ± 0.02 as many colonies on YP-glucose + hygromycin B as there were on YP-glucose without hygromycin B. In controls cultured for 40 generations in YP-glucose + hygromycin, there were 1.03 ± 0.05 times as many colonies on YP-glucose + hygromycin B as there were on YP-glucose without hygromycin B. Second, we transformed *E. coli* DH5α with DNA extracted from 25 independent *C. albicans* pYM70 transformants, and the *E. coli* transformants were plated on LB-ampicillin media. Plasmids capable of conferring ampicillin resistance to *E. coli* were obtained from 14 of 25 (56%) *C. albicans* transformants.

Properties of *C. albicans* integrative transformants

To determine if a single copy of the *CaHygB* marker would confer hygromycin B resistance, *C. albicans* CAI4 was transformed with the *ACT1*-regulated integrating vector pAU34, pAU34-CaHygB, the *MAL2*-regulated integrating vector pAU15, or pAU15-CaHygB. The resulting transformants were isolated and purified on minimal media lacking uridine, and they were tested for growth on hygromycin B. All of the pAU34-CaHygB transformants tested grew well on YP-glucose + 600 μg hygromycin per ml and on buffered YNB-glucose + 1000 μg hygromycin B per ml, whereas the pAU34-transformed controls did not. Also, all of the pAU15-CaHygB transformants tested grew on inducing (YP-maltose) medium + 600 μg hygromycin B per ml but not on repressing (YP-glucose) medium + 600 μg hygromycin B per ml, whereas pAU15-transformed controls grew on neither medium (Fig 2).

Compatibility of *CaHygB* with other markers

Whether the *CaHygB* marker would confer resistance to nourseothricin was examined by testing *C. albicans* CAI4 transformed with pAU34-HygB, pAU15-CaHygB or pYM70 for growth on nourseothricin; none of these transformants grew on YP-glucose or buffered

YNB-glucose containing 200-600 μg nourseothricin per ml. Whether the *SAT-1* marker would confer resistance to hygromycin B was examined by testing *C. albicans* strain YPT1 (Roemer *et al*, 2003) for growth on hygromycin B; this strain did not grow on YP-glucose + 600 μg hygromycin B per ml or on buffered YNB-glucose + 1000 μg hygromycin B per ml (Fig 2). Also, whether pYM70 and *URA3*-containing plasmids were compatible in *C. albicans* was assessed by transforming *C. albicans* CAI4 with pYM70 and with pSEC4 [which carries the *C. albicans URA3* and *SEC4* genes (Mao *et al.*, 1999)]. *C. albicans* transformed with both plasmids grew well on YNB-glucose + hygromycin B, whereas controls transformed only with pYM70 did not grow in the absence of uridine (Fig 2).

Targeted disruption of *HIS1*, *LEU2* and *ARG4* in wild-type *C. albicans*

Since integration of single-copy vectors containing the *CaHygB* marker conferred hygromycin B resistance in *C. albicans* and since the *CaHygB* and *SAT-1* markers did not cross-react with each other, we reasoned that it should be possible to construct null mutants in wild-type *C. albicans* strains by disrupting one chromosomal allele of a gene of interest with the *CaHygB* marker and the other allele with the *SAT-1* marker. To test this hypothesis, we used fusion PCR to construct linear gene-targeting molecules consisting of the *CaHygB* or the *SAT-1* markers flanked by the 5' and 3' noncoding regions of *C. albicans* SC5314 *ARG4*, *HIS1* and *LEU2*, and we introduced the *CaHygB*-containing amplicons into *C. albicans* strains SC5314, WO-1, B311, CT98001-5001, CT98004-5004 and CT98009-5009. Transformants derived from each host strain were selected and purified on YP-glucose + hygromycin B, and clones in which one chromosomal allele of each gene of interest had been replaced by homologous targeting were identified by PCR. Next, these heterozygous mutants were transformed again with the corresponding *SAT-1*-containing amplicons, and the resulting transformants were selected and purified on YP-glucose + hygromycin B + nourseothricin. PCR and phenotypic analyses showed that the second allele of all three genes of interest had been replaced by homologous targeting in all 6 wild-type *C. albicans* strains, with homologous targeting frequencies of approximately 50-70 percent. For example, homologous replacement of the second chromosomal alleles of *ARG4*, *HIS1* and *LEU2* in *C. albicans* SC5314 was demonstrated by PCR in 6 of 10, 7 of 10 and 6 of 10 transformants, respectively. Disruption of both chromosomal alleles of these genes was verified by showing that all 6 *arg4* null mutants did not grow in the absence of arginine, all 7 *his1* null mutants did not grow in the absence of histidine, and all 6 *leu2* null mutants did not grow in the absence of leucine (Fig 3).

DISCUSSION

The objectives of this study were to generate a synthetic hygromycin B resistance gene that functions in *C. albicans* and to develop multicopy expression plasmids and gene targeting strategies that employ this new marker. The key findings were that (i) all of the *C. albicans* strains tested were inhibited by 600 μg of hygromycin B per ml of YPD and by 1000 μg of hygromycin B in YNB buffered to pH 7.0 and (ii) the synthetic *CaHygB* marker conferred hygromycin B resistance when it was expressed under the control of the *C. albicans TEF2* promoter in an *ars2*-containing plasmid and also when it was expressed under the control of the *C. albicans ACT1*, *MAL2* or *TEF2* promoters in linearized single-copy integrating

vectors or in linear gene-targeting constructs. One important finding is that hygromycin B could be used in minimal media only when the medium was buffered to neutral pH.

The *ACT1*-regulated expression plasmid pYM70 conferred hygromycin B resistance to *C. albicans* transformants. When we incubated pYM70-transformed *C. albicans* without hygromycin B for 40 generations, a substantial minority of the transformants lost their plasmids. Moreover, plasmids that could replicate in *E. coli* were recoverable from most pYM70-transformed *C. albicans*. We concluded from these results that pYM70 can replicate in *C. albicans* as episomes. However, that plasmids that replicated in *E. coli* could not be recovered from a substantial minority of *C. albicans* transformants suggests that that pYM70 either integrated into the genome of the *C. albicans* host strain (as might be expected for a plasmid containing substantial amounts of *C. albicans* genomic DNA) or underwent structural alterations [e.g., concatenation into large multimers (Goshorn *et al.*, 1992)] that resulted in limited abilities to replicate in *E. coli*. Nevertheless, the usefulness of pYM70 as an expression vector was shown in a recent study in which we used pYM70 to overexpress the *C. albicans CDR1*, *CDR2* and *MDR1* genes in a *C. albicans cdr1 cdr2 mdr1* null mutant. The resulting *C. albicans* transformants were more resistant to fluconazole and several other azole antifungals, and they had lower intracellular [^{3}H]-fluconazole concentrations than, did empty-vector controls (Basso *et al.*, 2010).

The observation that all uridine prototrophs obtained by transforming *C. albicans* CAI4 with pAU34-*CaHygB* or with pAU15-*CaHygB* were also resistant to hygromycin B established that integration of a single copy of the *CaHygB* marker into the genome was sufficient to confer hygromycin B resistance in *C. albicans*. Since this suggested that the *CaHygB* marker could be used for homologous gene disruption, a convenient fusion PCR method for generating gene targeting molecules containing the *CaHygB* and *SAT-1* markers was developed, and the resulting amplicons were used sequentially to disrupt both chromosomal alleles of the *ARG4*, *HIS1* and *LEU2* genes in 6 wild-type *C. albicans* strains, including three laboratory strains and three bloodstream isolates from fungemic patients. That homologous targeting was demonstrated at frequencies of approximately 50-70 percent among antibiotic-resistant transformants generated from 6 different wild-type *C. albicans* strains suggests that the strain construction method described in this report may be very useful for analyzing the importance of potential virulence-associated genes in multiple wild-type *C. albicans* strains.

In summary, we have shown that the synthetic *CaHygB* marker confers resistance to hygromycin B in single and multiple copies in multiple strains of *C. albicans*. An *ACT1*-regulated expression plasmid containing *CaHygB* as a selectable marker replicates in *C. albicans* and can be used to overexpress *C. albicans* genes (Basso *et al.*, 2010). Also, *arg4*, *his1* and *leu2* null mutants were constructed in 6 wild-type *C. albicans* strains by sequential disruption of both chromosomal alleles with fusion PCR products containing the *CaHygB* and *SAT-1* selection markers. The *CaHygB* marker described here should be a useful addition to the tools available for studying the important human pathogen *C. albicans*.

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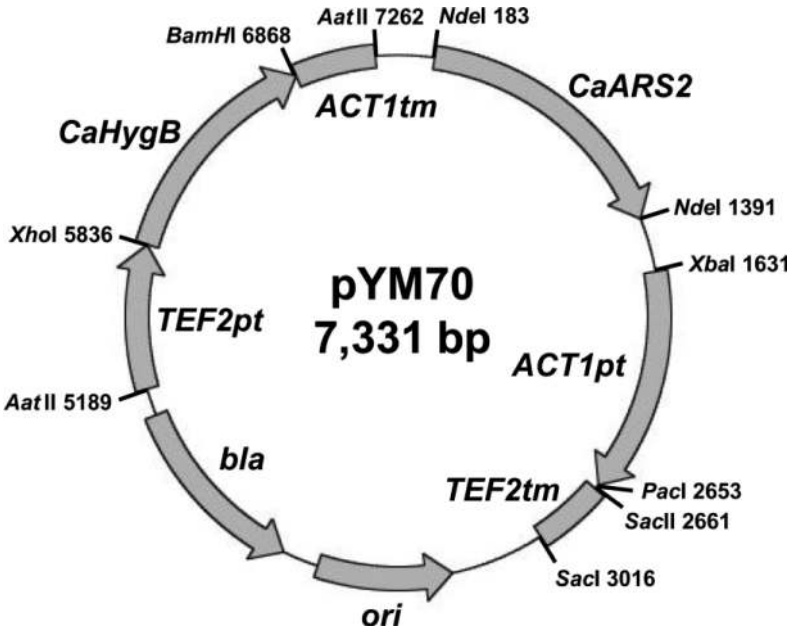

Figure 1.

Restriction map of the *ACT1*-regulated expression plasmid pYM70. Abbreviations: *CaARS2* = autonomously-replicating sequence; *ACT1pt* = *ACT1* promoter; *TEF2tm* = *TEF2* terminator; *ori* = origin of replication; *bla* = beta lactamase; *TEF2pt* = *TEF2* promoter; *CaHygB* = synthetic hygromycin B resistance gene; and *ACT1tm* = *ACT1* terminator.

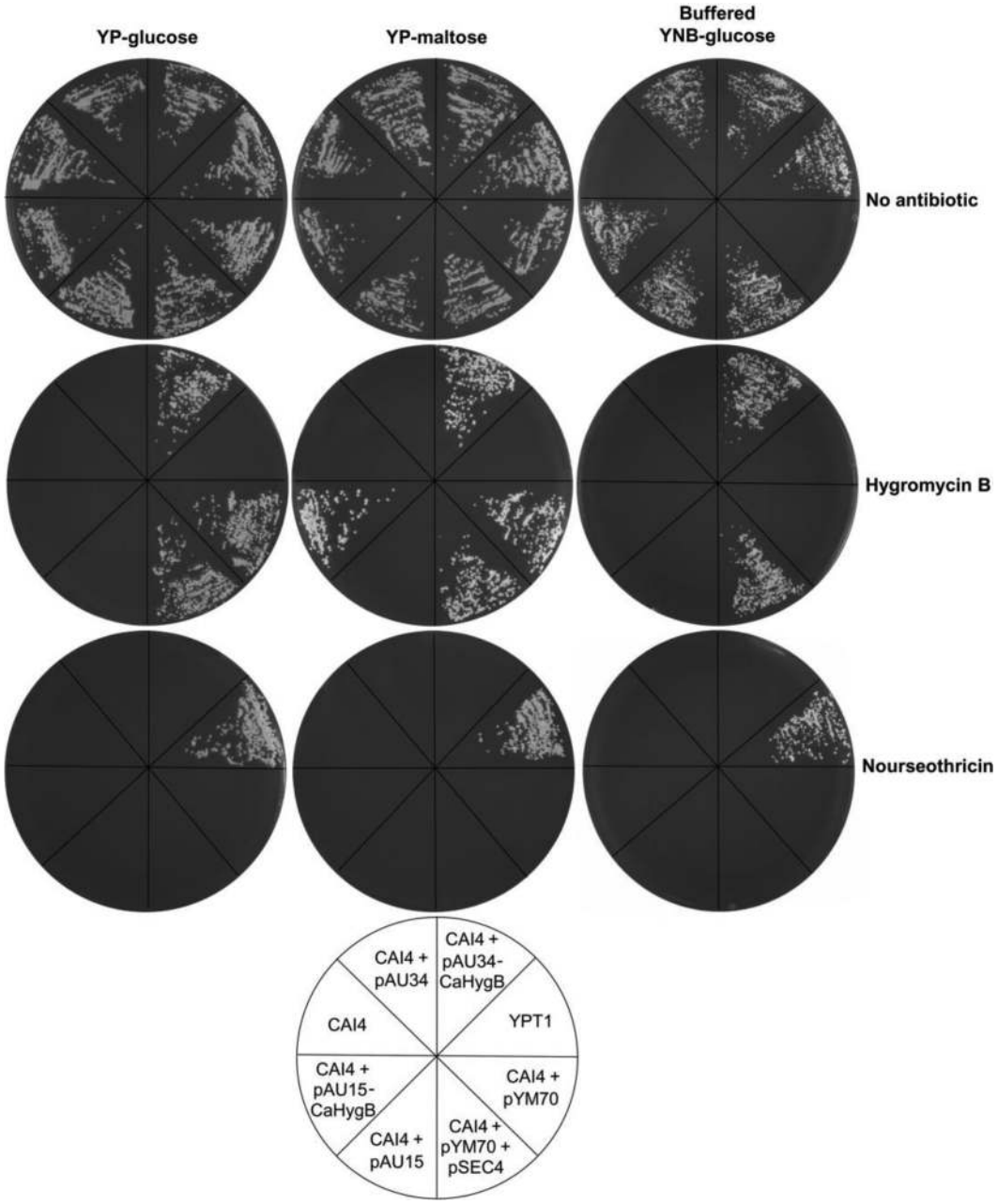

Figure 2.

Growth of *C. albicans* on hygromycin B and nourseothricin. *C. albicans* CAI4, *C. albicans* YPT1 (which contains the *SAT-1* marker), and *C. albicans* CAI4 transformed with pAU34, pAU34-CaHygB, pAU15, pAU15-CaHygB, pYM70, or both pYM70 and pSEC4 were incubated at 30°C for 48 h on solid YP-glucose, YP-maltose or buffered YNB-glucose containing either no antibiotic, hygromycin B, or nourseothricin. Strains in which *CaHygB* was expressed constitutively (pAU34-CaHygB, pYM70) or was induced by maltose (pAU15-CaHygB) grew in the presence of hygromycin B, and there was no cross-resistance between hygromycin B and nourseothricin.

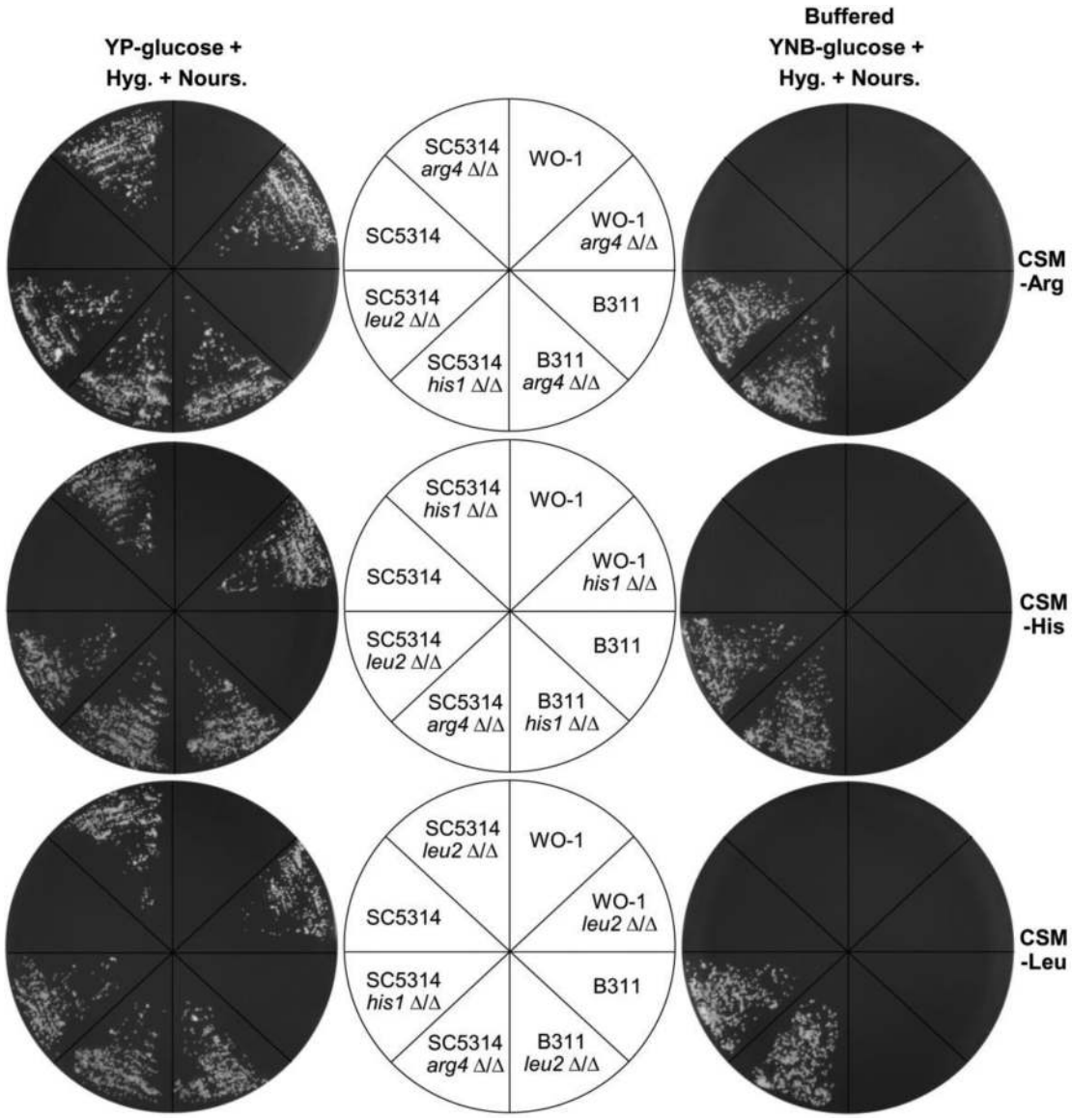

Figure 3.

Targeted disruption of *ARG4*, *HIS1* and *LEU2*. When the two *ARG4*, *HIS1* and *LEU2* alleles were replaced in *C. albicans* SC5314, WO-1 and B311 with the *CaHygB* and *SAT-1* markers, the resulting mutants acquired the ability to grow on rich medium (YP-glucose) + hygromycin B + nourseothricin. Homologous replacement of the genes of interest was verified by PCR (not shown) and by inability of the *arg4* Δ/Δ, *his1* Δ/Δ and *leu2* Δ/Δ mutants, respectively, to grow on minimal medium (buffered YNB-glucose) containing hygromycin B and nourseothricin without arginine (CSM-Arg), histidine (CSM-His) or leucine (CSM-Leu). Abbreviations: Hyg. = hygromycin B; Nours. = nourseothricin; CSM = complete synthetic medium.

Table 1

C. albicans strains used in this study

Strain	Parent Strain	Genotype	Reference or source
CAI4	SC5314	Δ*ura3*::*imm434*/Δ*ura3*::*imm434*	Fonzi and Irwin, 1993
SC5314	clinical isolate	Wild Type	Fonzi and Irwin, 1993
WO-1	clinical isolate	Wild Type	Sasnauskas *et al.,* 1992
B311	clinical isolate	Wild Type	Hasenclever and Mitchell, 1962
CT98001-5001	clinical isolate	Wild Type	This study
CT98004-5004	clinical isolate	Wild Type	This study
CT98009-5009	clinical isolate	Wild Type	This study
YPT1	CaSS1	*his3*::*hisG*/*his3*::*hisG leu2*::*tetR-GAL4AD-URA3*/*LEU2 YPT1*Δ::*HIS3*/*YPT1*	Roemer *et al.*, 2003

Table 2

Plasmids used in this study

Plasmid	Marker	Description	Reference
pAU34	*URA3*	*ACT1*-regulated integrating vector	Uhl and Johson, 2001
pAU34-CaHygB	*URA3*	*CaHygB* under control of *ACT1* promoter	This study
pAU15	*URA3*	*MAL2*-regulated integrating vector	Uhl and Johson, 2001
pAU15-CaHygB	*URA3*	*CaHygB* under control of *MAL2* promoter	This study
pSEC4	*URA3*	pYM1 derivative	Mao *et al.*, 1999
pYM70	*CaHygB*	pUC18 derivative with *CaHygB* under control of *TEF2* promoter and *ACT1* terminator	This study
pBSII-CaHygB	*CaHygB*	*CaHygB* marker under control of *TEF2* promoter and *MAL2* terminator	This study
SAT-1 cassette	*SAT-1*	*SAT-1* marker under control of *ACT1* promoter and *PCK1* terminator in pBluescriptII.	Roemer *et al.*, 2003

Table 3

Primers used in this study

Primer name	Sequence 5′ → 3′
Plasmid construction	
TEF2pt-5’	GTGGGTACCGACGTCGTATAGTGCTTGCTGTTCGATATT
TEF2pt-3’	GGTGGTGGTCTCGAGGATTGATTATATAAAATGTATACTTAGAAAA
ACT1pt-5’	GGTGGTTCTAGAAGAGCTATTAAGATCACCAGCCT
ACT1pt-3’	GGTGGTTTAATTAATTTGAATGATTATATTTTTTTAATATTAA
ACT1tm-5’	GGTGGTGGATCCGAGTGAAATTCTGGAAATCTGGA
ACT1tm-3’	GGTTCTAGAGACGTCATTTTATGATGGAATGAATGGGA
Gene deletion	
Upstream primers	
ARG4 5’	ATTTTGAAACAATGAATCGATGCTT
ARG4 3’	TCGCCCTATAGTGAGTCGTTATTAATTGATTATCTTGATAGCTGTTATG
HIS1 5’	GTGCCACTGTATACGCATTT
HIS1 3’	TCGCCCTATAGTGAGTCGTTATCGGTAGTTGGTGGTTAAGTAA
LEU2 5’	TTAGTTTCTATTATGGCCGTCAAT
LEU2 3’	TCGCCCTATAGTGAGTCGTGTTTTTTGGATATTGGTTTTAAAAGA
Downstream primers	
ARG4 5’	TTCCCTTTAGTGAGGGTTAATTTATAAATAGTCATATAATAATCACAGTAT
ARG4 3’	TGCAAACAAACAGGGGAAAA
HIS1 5’	TTCCCTTTAGTGAGGGTTAAAAGAAGTGATAGTTTCTCATAAATAT
HIS1 3’	TCAATTATGTTGATTAGCTACAGTCA
LEU2 5’	TTCCCTTTAGTGAGGGTTAAACAGTATATACAGTAGTTAGCATTT
LEU2 3’	TTTATACCACGTGGTGACGAA
Fusion primers	
ARG4 5’	CATAACAGCTATCAAGAATAATCAATTAATAACGACTCACTATAGGGCGA
ARG4 3’	ATACTGTGATTATTATATGACTATTTATAAAATAACCCTCACTAAAGGGAA
HIS1 5’	TTACTTAACCACCAACTACCGATAACGACTCACTATAGGGCG
HIS1 3’	ATATTTATGAGAAACTATCACTTCTTTTAACCCTCACTAAAGGGAA
LEU2 5’	TCTTTTAAAACCAATATCCAAAAAACACGACTCACTATAGGGCGA
LEU2 3’	AAATGCTAACTACTGTATATACTGTTTAACCCTCACTAAAGGGAA
HygB 5’	CTGGAATTGGCAAAGCAGCAGAAGCA
HygB 3’	TCAGCTGCTGTTTGGACTGATGGTTGT
Nours 5’	GTTCTCAGCATCCAATGTTTCCGCCA
Nours 3’	CTTCAAGTCTCGAACGAAACAGCGAT
Verification Primers	
ARG4 5’	GGTTCCTGGATTTGCGCAGCCTTATA
ARG4 3’	CGCGATTAGAACTTGTGGACCTATCCT
ARG4 3’A	CGTGTGATGTCAGTTGTTCAAGGTTGACT
ARG4 3’B	GCAGTTCCAAAGATTGAAGCGTCTTCGT
ARG4 3’C	GCTACATTACCCTCTGTTGCCACAAGCAT

Primer name	Sequence 5′ → 3′
ARG4 3'D	GTCTTTGGATCGGTAGTACTGTGGCA
HIS1 5'	AGGAAGGTCACAGCTTGGGGTTTGAT
HIS1 3'	GATTGGGTGGCCATATTGTTCAAGGACA
LEU2 5'	TGCCAGACATATGCAAGATGAAGGGT
LEU2 3'	ACCCACCATTACGCAGAAGAAAGTCA