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Generation of stable mutants and targeted gene deletion strains in *Cryptococcus neoformans* through electroporation

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Abstract

Cryptococcus neoformans is the etiologic agent of cryptococcal meningitis that causes more than half a million deaths worldwide each year. This capsulated basidiomycetous yeast also serves as a model for micropathogenic studies. The ability to make stable mutants, either via ectopic integration or homologous recombination, has been accomplished using biolistic transformation. This technical advance has greatly facilitated the research on the basic biology and pathogenic mechanisms of this pathogen in the past two decades. However, biolistic transformation is costly, and its reproducibility varies widely. Here we found that stable ectopic integration or targeted gene deletion *via* homologous replacement could be accomplished through electroporative transformation. The stability of the transformants obtained through electroporation and the frequency of homologous replacement is highly dependent on the selective marker. A frequency of homologous recombination among the stable transformants obtained by electroporation is comparable to those obtained by biolistic transformation (~10%) when dominant drug selection markers are used, which is much higher than what has been previously reported for electroporation when auxotrophic markers were used (0.001% to 0.1%). Furthermore, disruption of the *KU80* gene or generation of gene deletion constructs using the split marker strategy, two approaches known to increase homologous replacement among transformants obtained through biolistic transformation, also increase the frequency of homologous replacement among transformants obtained through electroporation. Therefore, electroporation provides a low cost alternative for mutagenesis in *Cryptococcus*.

Keywords

Cryptococcus neoformans; ectopic integration; dominant markers; electroporation; gene disruption; transformation

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Supplementary material

Supplementary material is available at *Medical Mycology* online (http://www.mmy.oxfordjournals.org/).

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

Introduction

Cryptococcus neoformans is the major etiologic agent of fungal meningitis [1-6]. This clinically important fungal pathogen also serves as a model for micropathogenic studies. There are complete genome sequences, several congenic pairs, robust mammalian and invertebrate host models, and various genetic tools available [6-12]. In particular, the introduction of biolistic transformation in early 1990s enables target mutagenesis (e.g., gene deletion) *via* homologous recombination, and it has been central to for fungal studies of *Cryptococcus* genes and their roles in pathogenicity [13-20].

Transformants obtained by biolistic introduction of DNA are generally stable (17.5% to 100%) [14,21]. The frequencies of homologous recombination vary widely depending on the gene and the strain background, but a frequency in the range of 1–10% is typical [13-19,22]. However, biolistic transformation is costly due to the requirement of the expensive Biolistic ® PDS-1000/He Particle Delivery System only available from BioRad. In addition, it requires a vacuum pump and a helium gas tank as well as other pricey consumables sold by BioRad (gold beads, macrocarriers, stopping screens, and the rupture disks). Repeated transformations are often necessary to obtain knockout mutants due to variability of this technique and frequencies of homologous recombination in this fungus. All these factors make the biolistic transformation inhibitory for resource-limited laboratories.

Other transformation systems have been explored, including protoplasting, *Agrobacterium*-mediated transformation (AMT), and electroporation. None of these attempts were successful for targeted mutagenesis [23-28].

Electroporation has been used in *Cryptococcus* research since early 1990s. All previous reports on electroporation were done in strains belonging to *Cryptococcus neoformans* var. *neoformans* (serotype D). Most of the electroporation reported earlier used nutritional auxotrophic markers such as *URA5* to select for transformants. The vast majority of transformants obtained through electroporation were unstable, and half of them tended to lose the introduced DNA even when they were grown on selective minimum medium [27-29]. The introduced DNA often acquires telomeric sequences from *Cryptococcus* genome and replicates autonomously [23,27,28]. Consequently, the introduced DNA is often maintained episomally without being integrated into the genome. A minor proportion of the transformants were stable and these transformants usually contained ectopic integration of the *URA5* sequences [23,27,28]. Homologous recombination by electroporation occurred at extremely low frequencies, in the range of 1/1000 to 1/100,000 [30-33]. Therefore, electroporation has not been used for targeted mutagenesis. Rather it is used to introduce DNA that is desirable to be episomally maintained, such as in a multicopy suppressor screen [34-36].

One reported study of *Cryptococcus* electroporation did not use auxotrophic markers [37]. Instead, a native *Cryptococcus* L41 ribosomal gene with a dominant mutation was used as a selective marker gene because the protein produced confers resistance to cycloheximide, an inhibitor of protein synthesis. The cycloheximide-resistant transformants obtained were stable, resulted from ectopic integration events [37]. However, whether homologous

recombination occurred in these transformants were not investigated. Given that bacterium-originated genes whose products confer resistance to nourseothricin (NAT) [35], geneticin (G418) [38,39], and hygromycin B (HYB) [40,41] are currently preferably used in *Cryptococcus* studies because these marker genes lack homology to the *Cryptococcus* genome, it will be important to know whether these dominant markers could increase the stability of introduced DNA in these transformants obtained by electroporation.

Unfortunately, electroporation in var. *grubii* (serotype A) has been considered extremely inefficient, and successful transformation by electroporation were previously done in var. *neoformans* (serotype D). A previous study obtained no transformants at all by electroporation in four strains in the *grubii* H99 genetic background [14]. However, *C. neoformans* var. *grubii* causes the vast majority of all cryptococcosis cases globally [3,6,42] and is in general more virulent than var. *neoformans* [43,44]. This current study is designed to examine whether electroporation with dominant selective markers (NAT and G418) can generate stable transformants in the H99 background, whether decreasing nonhomologous end joining by deletion of the *KU80* gene [45] or using a split marker strategy can increase the rate of homologous recombination, and whether electroporation can be practically used for targeted mutagenesis. We chose the *ADE2* locus for the primary investigation as deletion of the *ADE2* gene not only confers auxotrophy but also red pigmentation to the mutant colony that can be easily visualized [46]. We also included other genetic loci and a different genetic background in this study to demonstrate that this approach is not locus- or strain-specific.

Materials and methods

Strains and Growth Conditions

Strains used and generated in this study and their sources are listed in Table 1. Cells were maintained on YPD (1% yeast extract, 2% BactoPeptone, and 2% dextrose) agar medium at 30°C unless indicated otherwise.

Genomic DNA Preparations

Strains were grown in 50 ml YPD liquid medium at 30°C overnight with shaking. The cells were washed with sterile water, harvested by centrifugation, and frozen at −80°C. The cells were then lyophilized overnight and then broken into fine powder by glass beads. DNA was then purified from the powder using the CTAB protocol as described previously [47].

Generation of gene deletion constructs

To generate deletion constructs by triple-joint polymerase chain reaction (TJ-PCR) with the intact NAT marker, 5′ and 3′ flanking sequences (~1 kb each) of the gene of interest were amplified using the genomic DNA extracted from the wild-type H99 strain. Meanwhile, the NAT marker (~1.8 kb) that contains the *Cryptococcus ACT1* promoter and *TRP1* terminator was amplified using the pPZP-NATcc as the template [48]. Similarly, the G418 marker (~2.1 kb) was generated based on the plasmid pPZP-NEO1 [48]. The three products from the first round of PCR were then joined together to generate the deletion construct with the NAT marker flanked by the 5′ and 3′ flanking sequences of the gene of interest at either side

(Fig. 1, left panel) by a triple-joint overlap PCR. Primers used are listed in Supplemental Table 1.

To generate deletion constructs by double-joint PCR (DJ-PCR) with NAT split markers, 5′ and 3′ flanking sequences (~1 kb each) of the gene of interest were amplified using the genomic DNA extracted from the wild type H99 strain. Meanwhile, the first 1.2 kb of the NAT marker (5′-NAT) and the last 1.2 kb of the NAT marker (NAT-3′) with ~600 bp overlap were amplified using the pPZP-NATcc as the template [48]. The product of the 5′ flanking sequence connected with 5′-NAT was produced by a double-joint overlap PCR. Similarly, the 3′ flanking sequence was connected with 5′-NAT (Fig. 1, right panel).

The DNA fragments (5F-NAT-3F, 5F-G418–3F, or 5F-5′NAT + NAT3′-3F) were then introduced into *Cryptococcus* yeast cells by electroporation as described below.

Electroporation of Cryptococcus

Electroporation of *Cryptococcus* was performed using previously described method with minor modifications [29,49,50]. *Cryptococcus* yeast cells were grown in 30 ml of YPD medium at 30°C with shaking overnight. Cells were then diluted with YPD medium to A_{600} of 0.3 to a final volume of 100 ml. The cells were cultured for additional three hours to reach a cell density between A_{600} of 0.6 to 1.0. Cells were harvested by centrifugation at 4,000 × g for 5 min. The cells were then washed twice in 50 ml EB buffer (10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 270 mM Sucrose) and then resuspended in 50 ml of EB buffer with 1mM DTT. After 30–60 min of incubation on ice, cells were harvested, washed once with 50 ml of EB buffer, and then resuspended in 300 μl of EB buffer. The cell suspension (45 μl) was mixed with 5 μl of DNA (~100–400 ng), placed in a 0.2-cm electroporation cuvette, and transformed by electroporation using a BioRad gene pulser (0.45 kV, 125 μF, 400–600Ω) or an Eppendorf multiporator using the prokaryotic cell setting according to the manufacture's instruction. The electroporated cells were then suspended with 1 ml of YPD medium and incubated at 30°C for 90 min before being plated to appropriate selective media (YPD supplemented with either NAT at 100 μg/ml or G418 at 200 μg/ml). Transformants became apparent after 2 days of incubation at 30°C.

Transformants stability testing

Transformants from selective media were picked and transferred to master YPD plates. The cells were incubated at 30°C for 2 days and then replicated on fresh YPD agar medium. After fifth passage, cells were replicated on YPD solid medium with the appropriate antibiotic to examine the stability of the transformants. Colonies that showed spotty growth on the selective medium compared to those on non-selective YPD medium were scored as unstable. Colonies that grew similarly robustly on selective and nonselective media were scored as stable transformants.

Genetic cross

Mating partners were co-cultured together on V8 medium and incubated at 22°C in the dark for 2 weeks. Spores were microdissected using a dissection microscope. The mating type of the progeny was confirmed by crossing with reference strains as previously described [51].

Results

The stability of transformants obtained by electroporation is highly dependent on the marker used and targeted gene deletion can be achieved through electroporation

To examine the stability of DNA fragments containing the dominant NAT or G418 selective markers introduced into H99 by electroporation, we first generated the *ADE2* gene deletion constructs by triple-joint overlap PCR with the NAT or the G418 marker bordered by the 5′ and 3′ flanking sequences of the *ADE2* gene as depicted in Figure 1 (left panel). These DNA fragments were then electroporated into H99, and the transformants were selected on YPD agar medium supplemented with either NAT or G418. After 2–3 days of additional incubation, transformants were transferred to nonselective YPD medium, passaged on YPD medium five times, and then replicated on selective medium to examine their stability. Colonies that showed spotty growth on the selective medium were considered unstable (Fig. 2), while transformants with robust growth of the whole colonies on the selective medium were considered stable (Fig. 2, pointed by the arrows). We found that the frequency of stable transformants using NAT or G418 dominant drug markers varies (Table 2) consistent with previous findings using electroporation (100% stability with the cycloheximide resistant marker in one study [37] and about 10% stability with the *URA5* marker [29]).

We then examined if any of the stable transformants were *ade2*Δ mutants resulting from the homologous replacement of the *ADE2* gene by the marker. Two out of the 140 stable transformants obtained using the G418 marker turned red (Fig. 1), and the two transformants were also auxotrophic unable to grow on the YNB minimal medium, as expected for *ade2* mutants (Fig. 3). As ectopic integration or episomal maintenance of the introduced DNA fragment will generate transformants capable of growing in the absence of adenine, the absence of growth of these transformants in the minimal medium further supports that the introduced DNA likely replaced the *ADE2* gene in the genome. The replacement of the *ADE2* gene by the marker in these transformants was further confirmed by diagnostic PCR (Fig. 4). Taken together, the data indicate that all these red transformants are indeed *ADE2* deletion mutants as a result of homologous replacement of the *ADE2* gene by the marker.

Disruption of Ku80 increases the frequency of homologous recombination among the transformants

Ku proteins are important for rejoining broken DNA ends by nonhomologous end-joining pathways [52]. Reducing or abolishing Ku protein activities lowers nonhomologous end joining events while promoting homologous recombination. Such changes occur naturally in meiotic cells in both yeasts and animals [53-55] and have been used to increase homologous recombination for target mutagenesis in *Cryptococcus* by biolistic transformation [45].

To examine if deletion of the *KU80* gene also increases the frequency of homologous recombination in the transformants obtained through electroporation, we electroporatively transformed the *ku80*Δ mutant in the H99 background with the *ADE2* deletion construct. Because the *KU80*Δ gene in that strain was replaced by the G418 marker [45], only the construct made with the NAT marker (5F-NAT-3F) was used in the electroporation. Out of eight stable transformants, six were *ade2*Δ mutants as confirmed by their phenotypes (red

pigment and auxotrophy) and by PCR analysis as described earlier (Fig. 3; Table 2). Thus the frequency of homologous recombination among the stable transformants in the *ku80* mutant background is high, similar to what is observed among transformants obtained with biolistic transformation.

The split marker strategy improves the rate of homologous replacement among stable transformants

The *ADE2* gene deletion construct used earlier was generated with a triple-joint PCR and it is one DNA fragment containing an intact marker gene bordered by the *ADE2* flanking regions (Fig. 1, left panel). Previous studies in other fungi and in *Cryptococcus* have demonstrated that a split-marker technique can increase homologous recombination events among transformants obtained by biolistic bombardment [21,56,57]. The split marker construct is generated by a double-joint PCR (Fig. 1, right panel) and is composed of two DNA fragments: one contains the 5′ flanking sequences of the *ADE2* gene and the 5′ part of the NAT marker and the other contains the 3′ flanking sequences of the *ADE2* gene and the remaining part of the NAT marker with some overlap sequences.

We introduced the split marker construct into both the wildtype H99 strain and the *ku80* strain by electroporation. Out of 11 stable transformants obtained in the H99 strain, one is an *ADE2* gene deletion mutant. Not surprisingly, two out of the two stable transformants obtained in the *ku80* strain are the *ade2*Δ mutants. Our data indicate that split-marker strategy can indeed increase the frequency of homologous replacement. However, the gain would be minimal when the *ku80* strain is used.

Deletion of other genes in both H99 background (var. *grubii*) and XL280 background (var. *neoformans*) can also be achieved by electroporation

We next examined whether this technique can be applied to other genetic loci or other strain background. We decided to test this in H99 *ku80* mutant of an uncharacterized gene *CNAG2526*. Indeed, we successfully obtained a knockout mutant based on PCR screening (Fig. 4). Genetic crosses indicated a pattern of single-gene Mendelian segregation of the marker and also the creation of progeny with only *CNAG2526* deletion in either mating type *a* or mating type α background (Table 1). To examine if this technique could also be applied to other strain background, we deleted the gene *RZE1* in the H99 wildtype background (var. *grubii*) and in the XL280 wild-type background (var. *neoformans*). One out of 29 stable transformants screened in H99 background was a knockout mutant and one out of 19 stable transformants screened in XL280 background was a knockout mutant (Table 1). We also used this technique to generate ectopically integrated fluorescent protein fusions with Fad1 and Fas1 selected with the G418 resistant marker in the XL280 background [58] (Table 1). About 10% (n = 50) and 12% (n = 50) of the transformants are genetically stable.

Discussion

The frequency of homologous integration is largely predetermined by the particular fungal species, although it can be affected by the genetic locus. For example, high frequencies of homologous integration are observed in *Saccha-romyces cerevisiae* and *Aspergillus nidulans*

[59-61]. However, many other fungi, including *Neurospora crassa* [62], *Coprinus cinereus* [63], *Ustilago maydis* [64], and *Cryptococcus neoformans* predominantly have ectopic integration events. In *Cryptococcus*, homologous recombination typically occurs at the frequency of 1–10% based on targeted gene deletion using biolistic transformation. It was proposed that electroporation cannot deliver sufficient DNA to the nucleus for efficient integration into the genome [22], let alone homologous recombination. Although more transformants obtained by electroporation were unstable compared to biolistic transformation, we found that the frequency of homologous replacement among the stable transformants obtained by electroporation is comparable with those obtained by biolistic transformation. In practice, elimination of unstable transformants after several passages on nonselective media will enable similar screening of stable transformants for correct integration of the introduced DNA. Furthermore, the disruption of the *KU80* gene or the adoption of the split-marker strategy can increase the frequency of homologous integration among transformants obtained (Table 2). In cases where the *ku80* mutant strain is used for electroporation, the *ku80* mutation can be easily separated from the mutations of the gene of interest by backcrossing selected transformants with a wildtype strain (Table 1), as reported previously [45].

In summary, our study has demonstrated that electroporation can provide an alternative low cost approach for targeted mutagenesis in *Cryptococcus neoformans*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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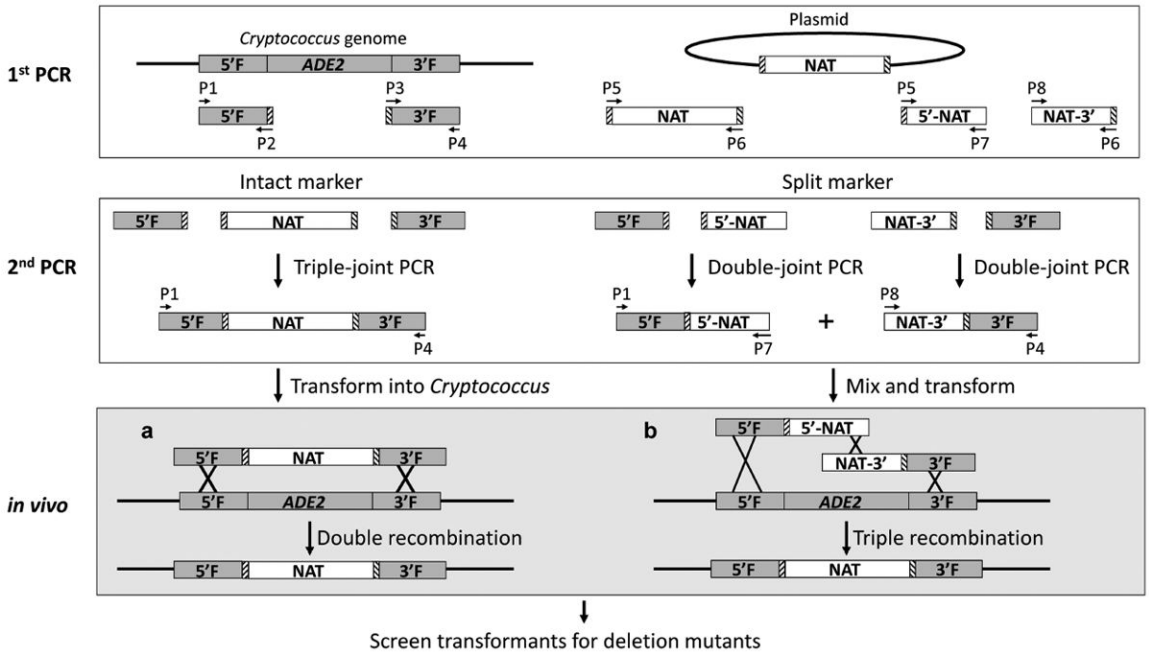

Figure 1.

A schematic diagram showing the procedure of generating the *ADE2* gene deletion constructs by a triple-joint polymerase chain reaction (PCR) or a double-joint PCR and transforming the constructs into *Cryptococcus* cells. During the first round of PCR, 5′ and 3′ flanking regions (5F and 3F) about ~1 kb each of the *ADE2* gene were amplified using primers sets P1+P2 and P3+P8, respectively. The NAT marker was amplified using primers P5 and P6. The 5′ and 3′-NAT-split markers (5′-NAT and NAT-3′) were amplified using primer sets P5+ P7 and P8+P9, respectively. During the second round of PCR, three different overlap PCR products were generated. The first product contained the NAT marker flanked by the 5′and 3′-flanking regions of the *ADE2* gene and was generated using primers P1 and P4 by a triple-joint PCR. The second product contained the 5′ flanking region of the *ADE2* gene connected with the first 2/3 of the NAT marker and was generated using primers P1 and P7 by a double-joint PCR. The third product contains the last 2/3 of the NAT marker connected with the 3′ flanking region of the *ADE2* gene and was amplified using primers P8+P4 by a double-joint PCR. The triple-joint PCR product or the mixed double-joint products were introduced into *Cryptococcus* cells by electroporation. Homologous recombination will lead to replacement of the *ADE2* gene by the selective marker. The boxes with slashes bordering the NAT marker indicate the regions complement to the primers P2 and P3 that were used to amplify the flanking sequences of the *ADE2* gene.

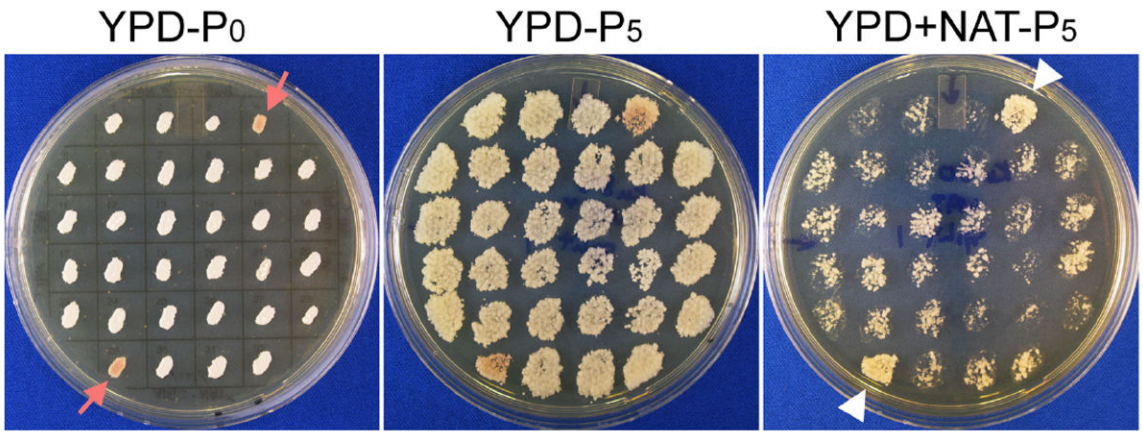

Figure 2.

The stability test of transformants obtained by electroporation. Transformants were transferred to a gridded YPD agar plate (left panel, YPD-P_0), passaged onto YPD solid medium for 5 consecutive times (YPD-P_5), and examined for their resistance to the appropriate antibiotic on selective agar medium. After fifth passage, transformants with spotty growth on the selective medium (shown here YPD+NAT-P_5 on the right panel) compared to that on nonselective YPD medium (YPD-P_5 on the middle panel) were considered unstable. Transformants that grew robustly on the selective medium as they grew on nonselective medium were considered stable (pointed by the white triangles on the right image on YPD+NAT). This particular original master plate (YPD-P_0) shown here have two colonies turned red, indicating of an *ade2* mutation (pointed by red arrows in the left image). These red transformants grew robustly on the YPD+NAT medium, indicating that these transformants are stable and the construct likely has been integrated into the genome and disrupted the *ADE2* gene.

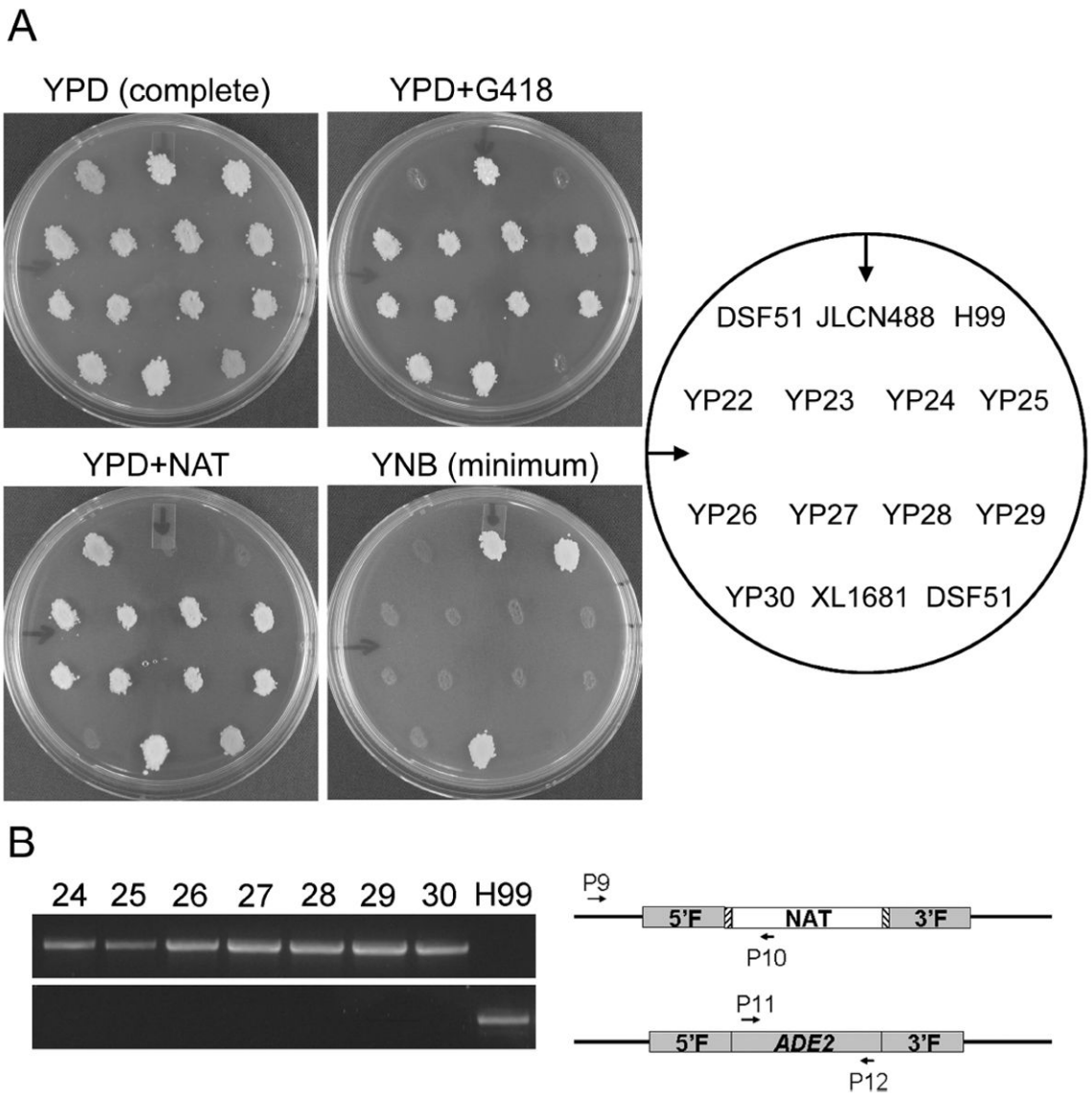

Figure 3.

Phenotypical and polymerase chain reaction (PCR) confirmation of selected transformants for the replacement of the *ADE2* gene by the marker. (A) The selected transformants YP22-YP29 and their parental strain JLCN488, transformant YP30 and its parental strain H99, and two control strains DSF51 (for ade2 and NAT resistant) and XL1681 (for NAT and G418 double resistant) were cultured on YPD agar medium at 30°C for 1 day and then replicated onto YPD medium for growth control. The colonies were also replicated onto YPD+G418 or YPD+NAT media to examine the presence of the G418 or the NAT marker. The colonies were replicated onto YNB minimum medium to examine auxotroph. Photographs were taken after 2 days of incubation at 30°C. (B) Genomic DNA extracted from red strains and the wild type strain H99 was used as the control. Only results of strains YP24-YP30 and H99 are shown here. The upper panel shows PCR amplicons using the primer P9 that localizes to the genomic region upstream of the 5′ flanking regions of the *ADE2* gene and the primer P10 that localizes in the NAT marker of the introduced construct. Only correct

integration of the construct will yield positive amplicons of 1.73 kb (DNA ladders not shown in the figure). The lower panel shows PCR amplicons using primers P11 and P12 that localize to the coding region of the *ADE2* gene. Strains with the *ADE2* gene present will yield positive amplicons of 1.70 kb.

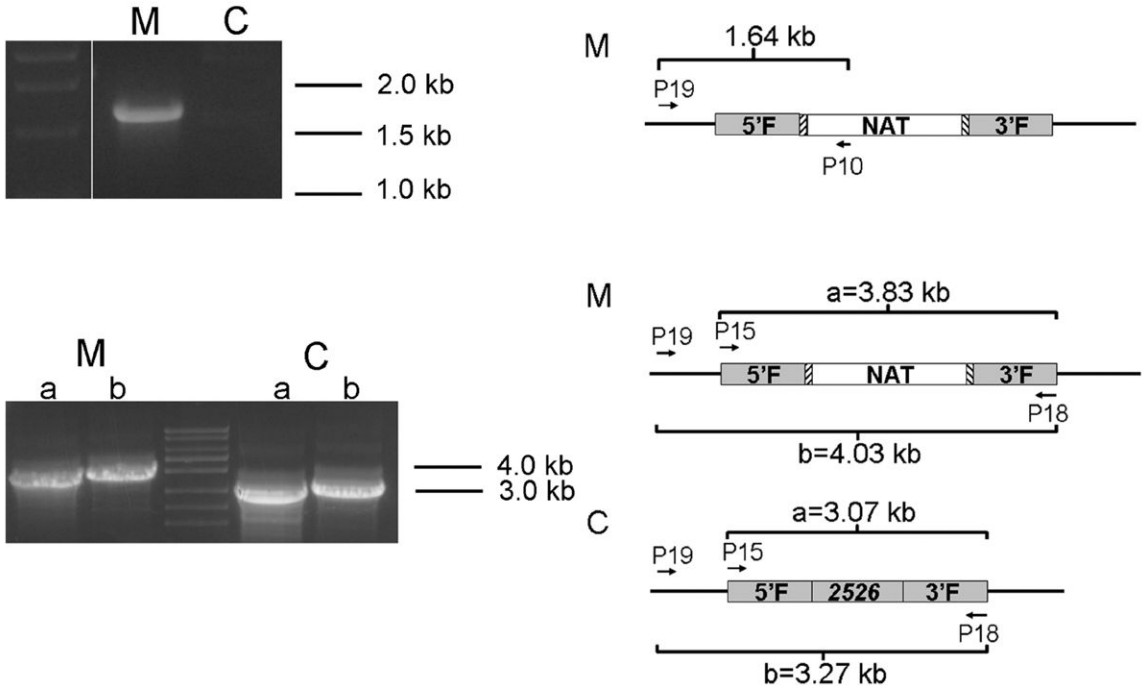

Figure 4.

Polymerase chain reaction (PCR) confirmation of homologous replacement of the *CNAG2526* gene in the stable transformant (XL1685). Genomic DNA extracted from XL1685 (labeled as M) and the control strain JLCN488 (labeled as C) was used as template. The upper panel shows PCR amplicons using the primer P19 that localizes to the genomic region upstream of the 5′ flanking regions of the *CNAG2526* gene and the primer P10 that localizes in the NAT marker of the introduced construct. Only correct integration of the construct will yield positive amplicons of 1.64 kb. The lower panel shows PCR amplicons using primer sets P15+P18 and P19+P18 that localize to the flanking region of the *CNAG2526* gene. Strains with the *CNAG2526* gene replaced by the NAT marker will yield positive amplicons of 3.83 kb and 4.03 kb respectively, while the control with the wild type allele of the *CNAG2526* gene will yield positive amplicons of 3.07 kb and 3.27 kb, respectively. (The expected sizes of the fragments were confirmed by longer electrophoresis.)

Table 1

Strains used in this study.

Strain name	Genotype	Source and comments
H99	Serotype A wild type	[65]
JLCN488	*MATα cku80*::*NEO*^{r}	[45]
XL1681	*MATα DsRED-NEO*^{r} *znf2*::*NEO*^{r} *ZNF2-NAT*^{r}	this study
DSF51	*MATα znf1*::*NAT, ste12*::*URA5, ade2*	this study
YP22	*MATα cku80*::*NEO*^{r} *ade2*::*NAT*^{r}	this study
YP23	*MATα cku80*::*NEO*^{r} *ade2*::*NAT*^{r}	this study
YP24	*MATα cku80*::*NEO*^{r} *ade2*::*NAT*^{r}	this study
YP25	*MATα cku80*::*NEO*^{r} *ade2*::*NAT*^{r}	this study
YP26	*MATα cku80*::*NEO*^{r} *ade2*::*NAT*^{r}	this study
YP27	*MATα cku80*::*NEO*^{r} *ade2*::*NAT*^{r}	this study
YP28	*MATα cku80*::*NEO*^{r} *ade2*::*NAT*^{r}	this study
YP29	*MATα cku80*::*NEO*^{r} *ade2*::*NAT*^{r}	this study
YP30	*MATα ade2*::*NEO*^{r}	this study
XL1685	*MATα cku80*::*NEO*^{r} *CNAG2526*::*NAT*^{r}	this study
XL1690	*MAT*a *cku80*::*NEO*^{r} *CNAG2526*::*NAT*^{r}	this study
XL1687	*MATα CNAG2526*::*NAT*^{r}	this study
XL1688	*MAT*a *CNAG2526*::*NAT*^{r}	this study
XL280#	Serotype D wildtype	[66]
NC01#	*MATα rze1*::*NAT*^{r}	this study
NC12	*MATα rze1*::*NAT*^{r}	this study
LW516α #	P_{FAD1}-*FAD1-mCherry*::*G418*^{r}	
LW728α #	P_{FAS1}-*FAS1-mCherry*::*G418*^{r}	[58]

strains in the XL280 genetic background. All others are in the H99 genetic background.

Table 2

Transformant stability and homologous replacement based on *ADE2*.

Strain	Construct	Total transformants	Stable transformants	Homologous replacement
Wild type H99	5F-NAT-3F	129	15	0
	5F-5′-NAT + NAT-3′-3F	49	11	1
	5F-G418-3F	164	140	2
ku80	5F-NAT-3F	102	8	6
	5F-5′-NAT + NAT-3′-3F	40	2	2