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Research Article

A high-efficiency method to replace essential genes with mutant alleles in yeast

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Abstract

Temperature-sensitive (TS), internally deleted and truncated alleles are important tools to facilitate the characterization of essential genes. We have developed a straightforward method to replace a wild-type gene with a mutant allele at the endogenous locus. This method is an efficient alternative to the two-step method for integration of alleles that are compromised in function or contain multiple mutations. A strain is constructed that has the essential gene of interest disrupted by a selectable marker. Strain viability is maintained by a plasmid carrying a copy of the essential wild-type gene and the *ADE3* gene. The mutant allele is cloned into an integratable vector carrying a selectable/counter-selectable marker, such as *URA3.* The plasmid is linearized and transformed, directing integration to the 5′ or 3′ region flanking the essential open reading frame (ORF). Transformants that have integrated the mutant gene at the endogenous locus can lose the autonomous plasmid carrying the wild-type copy of the essential gene and the *ADE3* gene. These transformants are identifiable as white sectoring colonies, display the mutant phenotype and may be characterized. An optional second selection step on 5-fluoroorotic acid (5-FOA) selects for popouts of the integrating vector sequences, leaves the mutant allele at the endogenous locus, and recycles selectable markers. We have used this method to integrate a TS allele of *SPC110* that could not be integrated by standard methods. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords: temperature-sensitive; gene replacement; integration; plasmid shuffle; conditional allele; counterselection

Introduction

Mutant alleles are important tools to provide insight into the functions of essential genes. A number of simple and high-efficiency methods have been developed for the replacement of non-essential genes with mutant alleles in yeast (Struhl, 1983; Szent-Gyorgyi, 1996). However, when working with essential genes, a classic two-step gene replacement method has been the main method used, since a functional copy of the gene in question must be present at all times to maintain strain viability (Scherer and Davis, 1979; Winston *et al*., 1983; Boeke *et al*., 1987; Rothstein, 1991). The basic strategy is to introduce the mutant allele by linearizing an integratable plasmid containing the mutant allele and *URA3*, a selectable/counter-selectable marker. Linearization directs integration to the endogenous locus of the gene, resulting in a tandem array of the wild-type and mutant allele flanking the *URA3* marker. After counter-selection on 5-FOA, the strains carrying the mutant allele are then identified by screening.

There are a few notable drawbacks to the two-step approach. The screen for the mutant allele can be time consuming if the mutant does not display an obvious phenotype. Alternatively, one may have to screen through a large number of colonies before isolating the strain with the gene-replaced allele because the mutant allele is selected against if the mutant is less fit compared to wild-type. In this case, it may be extremely difficult

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to replace the wild-type with the mutant allele. Also, if mutations are spread through a significant portion of the essential ORF, any number of them may be lost during integration or in the recombination event required for growth on 5-FOA. Other gene replacement strategies will work in many cases, but they are susceptible to gene conversion because wild-type sequence is present at the site of integration (Shortle *et al*., 1984; Erdeniz *et al*., 1997).

We have developed a high-efficiency method that overcomes these difficulties and allows replacement of an essential gene with any non-lethal mutation.

Materials and methods

Media

YPD medium and SD medium were made as described (Sherman *et al*., 1986). SD complete is SD medium supplemented with 50 µg/ml adenine, 25 µg/ml uracil, 100 µg/ml tryptophan, and 0.1% casamino acids. SD-tryptophan is SD complete lacking tryptophan. SD-uracil low adenine is SD complete lacking uracil and contains 5 µg/ml adenine. 5-FOA medium is SD complete with 100 µg/ml uracil and supplemented with 1 mg/ml 5-FOA (Boeke *et al*., 1987). YP glycerol medium is YPD medium with 2% glycerol substituted for glucose.

Plasmids

Plasmids used in this study are listed in Table 1. Plasmid pHS55 is the integratable version of plasmid pHS54. Plasmid pHS54 carries the *spc110-227* allele, which was recovered in the TS screen described previously(Sundberg and Davis, 1997).

Strains

The yeast strain used in this study is HSY2-12C. Its genotype is *MAT***a** *ade2-1oc ade3*Δ *can1-100 his3-11,15 leu2-3,112 lys2*Δ*::HIS3 spc110*Δ*::TRP1 trp1-1 ura3-1* carrying plasmid pHS26. It is a haploid isolate of HSY2 and is a derivative of W303(Geiser *et al*., 1993). The *Escherichia coli* strain used for plasmid preparations was XL-1 Blue (Stratagene).

Transformation and selection

pHS55 (1 µg) was linearized with SnaBI. The linearized DNA was transformed into the equivalent of 5 ml of a culture of HSY2-12C at 100 Klett units, using the LiAc TRAFO method (Schiestl and Gietz, 1989). The transformation reaction was plated on five SD-uracil low adenine plates and incubated at room temperature for 7 days. Sectored colonies were isolated and re-streaked on SD-uracil low adenine to obtain single, uniformly white colonies. Single white colonies from each sectored isolate were tested for growth on YP glycerol to eliminate isolates that were petite. The remaining white isolates were re-streaked on YPD in duplicate. One set was incubated at room temperature for 2 days to allow for recombination and loss of the *URA* and *TRP* markers at the *SPC110* locus, while the other set was incubated at 37 °C for 2 days to test temperature sensitivity. A sample from each room-temperature streak was struck heavily on 5-FOA plates and incubated at room temperature for 5 days. Single colonies were isolated from the 5-FOA plates and tested to see whether they had lost the ability to grow on medium lacking tryptophan.

PCR

Colony PCR using two pairs of primers was performed at three steps during the gene replacement.

Table 1. Plasmids used in this study

Plasmid	Parent plasmid	Relevant markers	Source or reference
pHS26	pTD29	SPC110 LYS2, ADE3, 2 µm origin	Geiser *et al*., 1993
pHS54	pHS32	spc110-227	Sundberg *and* Davis, 1997
pHS55	pRS306	spc110-227	Sundberg *and* Davis, 1997
pLI831	DK202	ADE3	Muller, 1996
pRS306		URA3, f1 origin	Sikorski *and* Hieter, 1989
pRS316		CEN6 ARSH4, URA3, f1 origin	Sikorski *and* Hieter, 1989

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Primer positions are illustrated in Figure 2d. Primer 1 anneals in the middle of the *TRP1* ORF and has the sequence 5′-TAATTTCACAGGTAGTTC-3′. Primer 2 is primer T7 with sequence 5′-TAATACGACTCACTATAGGG-3′. Primers 3 and 4 span the *SPC110* ORF and have the sequences 5′-TGCGCGGATATCTTAAGCAA-3′ and 5′-TTGA-AGATCAGTCTGCGTAGG-3′, respectively. Colony PCR was performed in 50 µl reactions containing 1 × GC buffer, 200 µM dNTPs, 0.5 µM each primer, and 0.5 µl Phusion™ DNA polymerase (Finnzymes). Reactions were cycled 30 times at 98 °C for 10 s, 50 °C for 20 s and 72 °C for 2 min, with a final extension step of 72 °C for 7 min. PCR products were separated on a 1% agarose gel with a 1 kb ladder (NEB).

Sequencing

Colony PCR was performed using primers 3 and 4 as described above. The 3 kb products were purified with the QIAquick® PCR purification kit (Qiagen). Sequencing was done with primer 5′-TGAAATCTGAACAGAGTA-3′.

Results and discussion

We have developed a new method for gene replacement that is based on a plasmid shuffle strategy (Bender and Pringle, 1991). The essential ORF of interest is replaced with any selectable marker except *URA3* in a diploid strain that is homozygous for the *ade2-1oc* mutation and *ade3*Δ. PCR cassette based knock-out strategies using kanamycin or hygromycin resistance genes have made this process simple and efficient (Wach *et al*., 1994; Goldstein and McCusker, 1999). The strain is then transformed with a plasmid containing the essential ORF with flanking upstream and downstream sequence (typically ∼500 bp 5′ and 3′), and the *ADE3* gene. The *ADE3* gene can be used for selection on medium lacking histidine if no *HIS* genes are compromised. If not, a selectable marker other than *URA3* must be included on the *ADE3* plasmid. We routinely use a plasmid carrying a 2 µm origin of replication, *ADE3* and *LYS2* for selection after transformation, which is made using plasmids pLI831, pTD29 and the essential gene of interest (Muller 1996). Selection for the *ADE3* plasmid is followed by sporulation and isolation of a haploid carrying the disrupted ORF and the *ADE3* plasmid. The strain must maintain the *ADE3* plasmid for viability, since it carries the only copy of the essential ORF. Due to the maintenance of *ADE3* in an *ade2-1oc* background, colonies of this strain are red, especially when supplied minimal amounts of adenine. This strain is called the 'plasmid shuffle strain' (Figure 1a).

Following strain construction, the mutant ORF with flanking sequence is cloned into an integratable vector containing the *URA3* gene, such as pRS306 (Sikorski and Hieter, 1989). The plasmid is then linearized with a unique restriction site in the 5′ or 3′ region of the essential gene. If a unique site is not available, one must be engineered. We generally use a unique site in the 3′ region. Transformation of the linearized plasmid into the plasmid shuffle indicator strain directs integration into the 3′ region of the essential gene, which is present at the endogenous locus or into the 3′ region of the copy of the gene in the *ADE3* plasmid (Figure 1b). Integration into the endogenous locus allows for loss of the *ADE3* plasmid, while integration into the *ADE3* plasmid makes its loss lethal, since it carries both functional copies of the essential gene. Consequently, when the transformation reaction is plated on SD-uracil low adenine plates, the former event will result in white sectored colonies, while the latter will give solid red colonies (Figure 1c). Solid white colonies are isolated from the sectored colony by re-streaking for single colonies. Loss of mitochondrial DNA can cause a white sectoring colony, but these colonies are easily identified by their inability to grow on medium with glycerol as the sole carbon source. The white colony able to grow on YP glycerol has the *URA3* marker flanked by the disrupted ORF and the mutant gene at the endogenous locus (Figure 1c). The mutant phenotype will be apparent at this stage. Plating on medium containing 5-FOA selects for recombination events that remove the *URA3* marker (Figure 1d). One of these events will remove the sole functional copy of the essential gene, resulting in a non-viable strain. The other event will result in a viable strain that carries the desired mutant allele at the endogenous locus.

As proof of principle, we integrated *spc110-227*, which is a temperature-sensitive allele of a gene encoding a spindle pole body component (Sundberg and Davis, 1997). This allele falls within the same complementation group as the *spc110-226*

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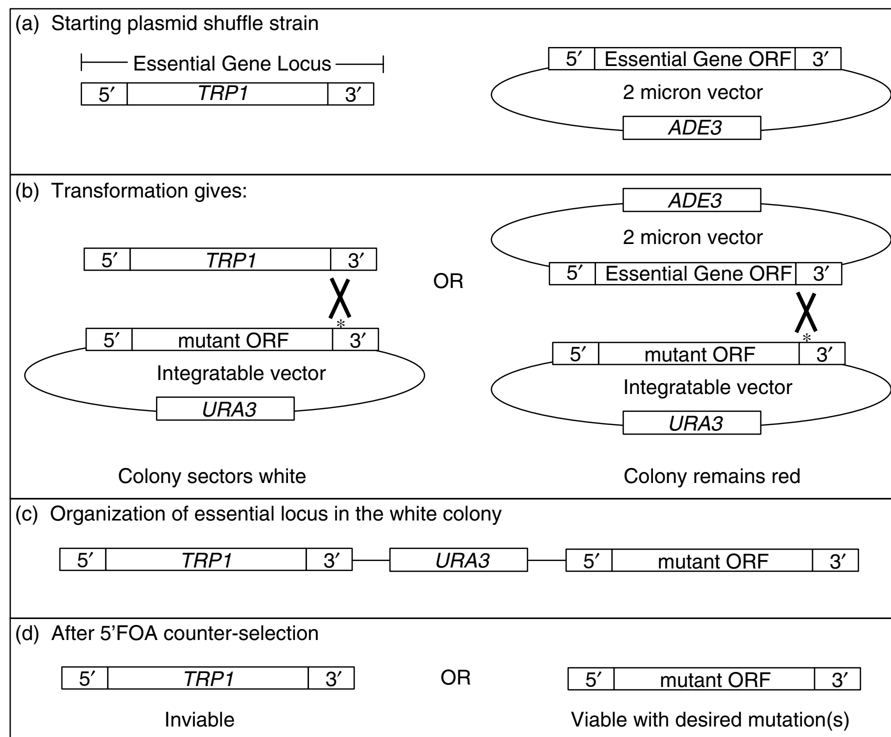

Figure 1. (a) The plasmid shuffle strain: a strain has the ORF of interest replaced with any selectable marker except *URA3*. In the example shown here, *TRP1* is used. The 5′- and 3′ regions flanking the ORF are indicated. A 2 μm plasmid carrying the wild-type gene is required to maintain strain viability. The 2 μm plasmid also carries the strain's sole copy of *ADE3*. The strain carries the *ade2-1oc* mutation and therefore remains red, since cells losing the 2 μm plasmid are not viable. (b) Integration: an integratable plasmid carrying *URA3* for selection and the mutant gene is linearized at a unique restriction site marked with an asterisk. Linearization directs integration into the 3′ region of either the endogenous locus or into the *ADE3* plasmid. (c) Organization of the essential locus: cells losing the *ADE3* plasmid are viable only if integration occurs at the endogenous locus, resulting in a sectored colony. The white colony has the deleted ORF, followed by *URA3* and the mutant gene at the endogenous locus. (d) Popout: the white colony is put on 5-FOA to select against the *URA3* marker. Recombination between the 3′ regions results in an inviable strain, while recombination between the 5′ regions results in a viable strain with the desired mutations

allele, which has defects in spindle assembly and cannot achieve a bioriented spindle (Yoder *et al*., 2005). Previous attempts to integrate the *spc110-227* allele by the classical two-step method had failed.

The plasmid shuffle indicator strain was made as described previously (Geiser *et al*., 1993). In this case, *TRP1* replaces the *SPC110* ORF and plasmid pHS26 is the *ADE3* plasmid. Linearization and transformation of 1 μg plasmid pHS55, which contains the *spc110-227* allele, yielded 715 transformants. Of those, 21 sectored white (Figure 2a). Sectored colonies were re-streaked to isolate single, uniformly white colonies. Seven of the isolates generated only white colonies unable to grow on YP glycerol, indicating that these colonies were petite and not of interest. All remaining 14 isolates were temperature-sensitive at 37 °C (Figure 2b). The 14 isolates were all able to generate colonies on 5-FOA that were also subsequently unable to grow on medium lacking tryptophan, confirming loss of the *spc110::TRP1* allele by recombination. We monitored this process by colony PCR (Figure 2c,d). Finally, all 14 isolates contained all of the mutations present in the *SPC110 ORF* in plasmid pHS55, as confirmed by sequencing. The *spc110-227* allele has seven mutations spanning approximately 350 base pairs. Five of the seven mutations (A2305G, T2405C, A2434G, G2452A and T2491A) result in the published amino acid changes (R784G, M802T, K812E, E818K and Y831N) (Sundberg and Davis,

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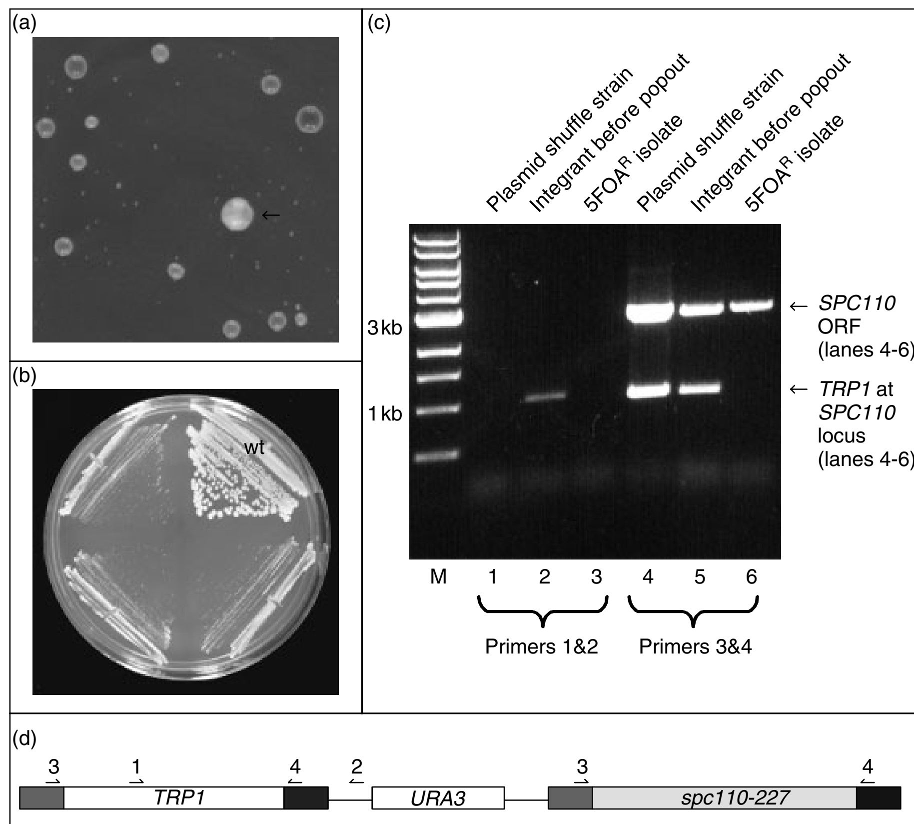

Figure 2. (a) Sectoring: a single transformation of the linearized integration vector gave 21 sectored colonies. A sample of transformants is shown. The arrow indicates a sectored colony. (b) Phenotype: temperature sensitivity of three representative white colonies compared to a wild-type (wt) strain. Strains were incubated at 37 °C for 2 days. (c) Colony PCR using two sets of primers was performed at three stages of the replacement. Lane 1, the plasmid shuffle strain is the template, using primers 1 and 2; lane 2, a *URA*$^+$ white isolate is the template, using primers 1 and 2; lane 3, a *TRP–URA–* 5-FOA isolate is the template, using primers 1 and 2; lane 4, the plasmid shuffle strain is the template, using primers 3 and 4; lane 5, a *URA*$^+$ white isolate is the template, using primers 3 and 4; lane 6, a *TRP–URA* isolate is the template, using primers 3 and 4. Lane M contains a 1 kb ladder. (d) Primer positions at the *SPC110* locus. The 5′ and 3′ regions on *SPC110* are in green and blue, respectively. The mutant *spc110-227 ORF* is in yellow

1997). The remaining two mutations (A2322G and A2673G) are silent. Base pairs and residues are numbered from the start of the *SPC110* ORF.

The increased efficiency of this method compared to the classic two-step method can be attributed mainly to the extent of selection against the mutant allele. In the two-step approach, mutations can be lost during integration because wild-type sequence is present at the site of recombination. Degradation of the ends of the linearized integratable vector increases this possibility. After selection for the integration event, one must allow sufficient cell divisions for a popout event to occur. Selection against the mutant allele occurs directly after recombination, progressively decreasing the fraction of the total cells with the mutant allele with each cell division. Depending on the severity of the selection, this may make the possibility of isolating the mutant very low. Our method minimizes these problems. During integration, mutations in the ORF of the mutant allele are not lost because wild-type sequence is not present at the endogenous locus. Gene conversion with the wild-type sequence on the autonomous plasmid is possible but this event is very infrequent, since we rarely see the plasmid shuffle strain sector on its own and this rare event must also result in a *URA*$^+$ colony for it to be picked up in our screen. After transformation, selection against the mutant allele only affects the extent of sectoring in the colony that

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arose from a cell that integrated the mutant allele at the endogenous locus. Even a colony containing an exceptionally compromised allele at the endogenous locus will eventually sector to an extent that can be seen, making this assay very sensitive. As soon as sectoring occurs, selection against the mutant allele cannot occur and no reversions are possible, since no wild-type sequence is present in the mutant white colony.

This method can be used as both a one-step and a two-step method. The white colonies display the mutant phenotype; consequently, any allele may be characterized after a single transformation and selection. The optional second popout step on 5-FOA can be done at any time and is useful when one needs to make the two selectable markers available. The method is also readily available to those already using a red/white screen for TS mutants. One only needs to convert recovered mutant alleles that are on ARS/CEN plasmids to integratable versions. Conveniently, it can also be modified for use in the *URA*-based TS screen, where a 2 μm or ARS/CEN plasmid contains *URA3* and the wild-type allele, and the ARS/CEN vector with the mutant allele has another selectable marker (Boeke *et al*., 1987). Essentially, integration is performed as described, but selection for the integrants into the genomic locus occurs on 5-FOA medium. If a popout of the integratable plasmid is desired, then the integratable vector must contain a selectable/counter-selectable marker other than *URA3*, such as *LYS2*.

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