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A two-step method for the introduction of single or multiple defined point mutations into the genome of *Saccharomyces cerevisiae*

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Abstract

The introduction of defined mutations into open reading frames (ORF) or non-translated regions of the genome is important to study of the structure–function relationship of amino acid residues in proteins or that of sequence motifs at the genome level. We describe a simple two-step method for the introduction of defined single or multiple point mutations into the genome of *Saccharomyces cerevisiae*. This method circumvents the need for plasmid-based mutagenesis and thus ensures homogenous expression of the gene of interest within the cell population. It is based on the introduction of a selectable marker downstream of the gene of interest. This marker is then amplified with a gene-specific primer that harbours the desired point mutation, creating a selectable marker-tagged mutant version of the gene of interest. The mutant fragment is then integrated into the genome of a wild-type strain through homologous recombination. Successive rounds of amplification of the mutant loci with primers that introduce additional point mutations upstream of existing mutations will generate multiple defined mutations within a single gene. As a proof of principle, we have employed this method to generate a temperature-sensitive mutant version of the plasma membrane ATPase, *pma1-7,* which bears two point mutations (Pro434Ala and Gly789Ser). Phenotypic analysis of a *pma1-7* haploid strain indicates that this allele has the same characteristics as the original *pma1-7* allele. It confers a temperature-sensitive growth phenotype and the newly synthesized Pma1-7 protein is unstable and rapidly degraded. Copyright © 2006 John Wiley & Sons, Ltd.

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Introduction

Site-directed mutagenesis is a powerful tool for studying structure–function relationships in proteins and DNA and to generate conditional alleles of essential genes. Traditionally, the method is applied to alter single nucleotides in DNA that has been cloned in a plasmid suitable for an *Escherichia coli*-based mutagenesis system (Sambrook *et al*., 1989). The plasmid is then transformed back into yeast to generate a strain that carries the mutated version of the gene of interest on a plasmid but lacks the corresponding wild-type locus in the genome to assess the function of the mutant allele.

To circumvent the need to first clone the gene of interest and subsequently select for the plasmid-borne auxotrophic markers, it is desirable to perform nucleotide exchanges at the genomic level, which is feasible in yeast due to the high efficiency of homologous recombination. Genomic expression of the altered allele is desirable, as it ensures defined copy number and homogeneous expression across the cell population and expression of the protein at endogenous levels. Methods to transfer alleles of a given gene from one strain background into another, or to directly modify the sequence of a wild-type gene to generate a desired point mutation, have previously been established but are frequently complicated. For example, some

of these protocols require a selectable phenotype of the desired point mutation (Moerschell *et al*., 1988; Rothstein, 1991), prior cloning of the gene of interest into a specialized plasmid (Barton *et al*., 1990; Scherer and Davis, 1979), require the use of specialized plasmids for the multi-step generation of a PCR-produced integration cassette (Erdeniz *et al*., 1997; Langle-Rouault and Jacobs, 1995), require long oligonucleotides (80–100 bp, e.g. for the 'delitto perfetto' method; Storici *et al*., 2001), or rely on the elimination of a selectable/counter-selectable marker that had been inserted into the gene of interest by direct repeat recombination, which limits their use to genes that are non-essential or to diploid cells (Gray *et al*., 2004, 2005; Szent-Gyorgyi, 1996).

To overcome some of these limitations, we describe here a cloning-free PCR-based allele replacement method that does not rely on the use of a counter-selectable marker or specialized plasmids and can be utilized to generate multiple defined mutations within the gene of interest, even if the gene is essential. As a proof of principle, we utilized the method to generate a temperature-sensitive mutant allele of the plasma membrane ATPase, *PMA1*, containing two point mutations. We show that this allele of PMA1, *pma1-7*, has the same phenotypic properties as the original *pma1-7* allele (Chang and Fink, 1995). It confers a temperature-sensitive growth phenotype and the mutant version of the protein is rapidly degraded when synthesized at 37 °C.

Materials and methods

Yeast strains and growth conditions

Yeast strains used in this study are derived from BY4741 or BY4742 (Winzeler *et al*., 1999) and are listed in Table 1. Strains were cultivated in YPD rich media [1% Bacto yeast extract, 2% Bacto peptone (US Biological Swampscott, MA), 2% glucose] or minimal media (Sherman *et al*., 1986). Yeast was transformed by lithium acetate (Ito *et al*., 1983).

Marker cassette-tagging and generation of a *pma1-7* allele

Tagging of the chromosomal locus of interest was performed by homologous recombination using the selectable marker gene from the template pFA6a–His3MX6 (Longtine *et al*., 1998). The marker gene was amplified by preparative PCR with the gene-specific targeting primers listed in Table 2.

Preparative PCR was performed in 3 × 100 µl reactions each containing 1× reaction buffer (10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$ and 0.001% gelatin), 200 µM dNTPs, 1 µM each primer, and 0.4 µl Taq DNA polymerase (Sigma Chemical Co., St Louis, MO) and 0.4 µl High Fidelity PCR Enzyme Mix (Fermentas, St. Leon-Rot, Germany). Reactions were cycled 30 times at 94 °C for 1 min, 55 °C for 1 min and 65 °C for 2 min, with a final extension step of 65 °C for 20 min. DNA from this PCR reaction was then precipitated by ethanol and resuspended in 30 µl water.

PCR fragment (3–5 µg) was used to transform the BY4742 (RSY1533) haploid wild-type strain. Correct integration of the marker cassette was confirmed by analytical colony PCR in a volume of 50 µl, using the same conditions as described above for preparative PCR. Analytical PCR products were precipitated with ethanol for subsequent restriction analysis or directly separated on a 1% agarose gel with a 100 bp ladder (Fermentas, St. Leon-Rot, Germany). Genomic DNA was isolated as described by Sherman *et al*. (1986) and used as template for preparative PCR reaction to introduce the desired point mutation.

Table 1. *S. cerevisiae* strains used in this study

Strain	Relevant genotype	Source or reference
YRS1533	BY4742; *MATα his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0*	EUROSCARF: Winzeler (1999), #4157
YRS2784	BY4741; *MAT***a** *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0*	EUROSCARF; Winzeler (1999), #4157
YRS2331	*MATα*; *his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 PMA1::His3MX6*	This study
YRS2783	*MAT***a**; *his3Δ1 leu2Δ0 ura3Δ0 met15Δ0 LYS2 pma1-G789S::His3MX6*	This study
YRS2779	*MATα*; *his3Δ1 leu2Δ0 ura3Δ0 lys2Δ0 MET15 pma1-P434A,G789S::His3MX6*	This study

Table 2. Primers used in this study

Name	Primer sequence (5′–3′)	Purpose
F1Pma1 cassette	ATAATTTTTG TCACATTTTA ATCAACTTGA TTTTTCTGGT cggatccccgggttaattaa	Forward primer for *PMA1::His3KanMX6* cassette
R1Pma1GFP	GATGAAATTT TAAGCAAACT CTAGTAGGAA ATAAGCGGCT gaattcgagctcgtttaaac	Reverse primer for *PMA1::His3KanMX6* cassette
Pma1G/A2365	AAGGGTGGTA TTATCCAAAA CTTC**A**GTGCT ATGAACGGTA TTATGTTCTT GCAAATTTCC	Forward first mutation
Pma1C/G1300	AGGCTTTCTT GAAGTCTTTG AAGCAATAT**G** CAAAGGCTAA GGACGCTTTG ACCAAGTACA	Forward second mutation
R1′Pma1	AAGGTGTGTG TGTGGATAAA ATATTAGAAT GACAATTCGA GATGAAATTT TAAGCAAACT	Reverse first and second mutation
Pma1-a	TTGATTGTTT TCATCGCTAT	Forward primer for *PMA1::His3KanMX6* cassette control
His5contr-r	TACGGGCGAC AGTCACATC	Reverse primer for *PMA1::His3KanMX6* cassette control
Pma1-e	GTCGAAGGTG CTACTGATGC	Forward first mutation control
Pma1-frev	TGGCTTACCG TTCATCAATC	Reverse first mutation control
Pma1-c	GGTCTGGACT GCTTGTTTCT	Forward second mutation control
Pma1-drev	CAAGTGTCTA GCTTCGCTAA	Reverse second mutation control

Sequences in upper case indicate homology to the genome of *S. cerevisiae* and those in lower case indicate homology to the PCR cassette employed. Point mutations are indicated in bold and underlined.

The presence of the point mutation in candidate colonies was confirmed by restriction fragment length polymorphism (RFLP) and DNA sequencing. Sequencing of PCR fragments was performed by Microsynth AG, Balgach, Switzerland.

Pulse-chase analysis of Pma1p

To determine the stability of newly synthesized mutant forms of Pma1p, cells were pulsed with radiolabelled amino acids for 5 min, chased for the time points indicated, and Pma1p was immunoprecipitated and analysed as described previously (Gaigg *et al*., 2005).

Results and discussion

A simple method to generate genomic point mutations

Although a number of methods to introduce point mutations into the genome of *S. cerevisiae* have been reported previously, none can be employed without the use of dedicated reagents, such as plasmids, selectable/counter-selectable markers, be suitable for the introduction of multiple point mutations, or would allow the isolation of point mutations that do not confer a specific phenotype. To overcome these limitations, we designed a generally applicable method that does not require special reagents or a scoreable phenotype for the resulting point mutation.

The general strategy of the method is outlined in Figure 1. It is based on a two-step introduction of the desired point mutation. In a first step, the gene of interest is targeted in the 3′ untranslated region (UTR) with a selectable marker, using standard integration techniques, e.g. as applied to the generation of gene deletions. In a second step, the marker-tagged gene is amplified using a gene internal primer that bears the desired mutation. The PCR fragment bearing the mutant version of the gene linked to the selectable marker is then transformed into a wild-type strain. Through homologous recombination, the mutant gene is then inserted at the wild-type locus, resulting in a replacement of the wild-type by the mutant locus. Transformants are then first screened for the presence of the selectable marker at the correct locus by colony PCR with a gene-specific primer. Positive colonies are then further screened for the presence of the point mutation, which may be present or has been eliminated during the recombination process. This last screen can be performed in different ways, depending on the precise nature of the desired mutation. For example (a), if the resulting mutation confers a growth phenotype it can directly be screened for. (b) In

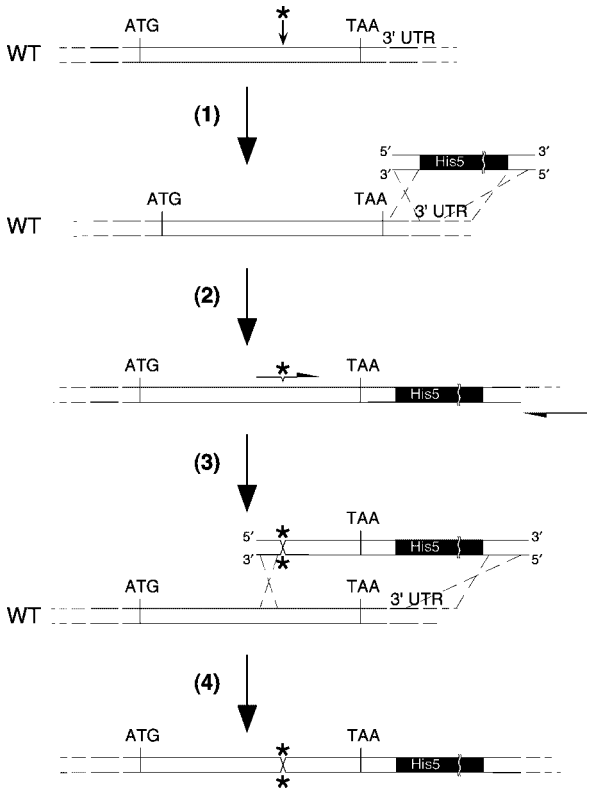

Figure 1. Outline of the mutagenesis strategy. To introduce a defined point mutation as indicated by the star (∗) into the gene of interest that is delineated by the start and stop codons, the gene is first (1) targeted at the 3′ UTR with a selectable marker; (2) genomic DNA from the resulting strain is then PCR amplified with a forward primer bearing the desired point mutation; (3) the resulting DNA fragment is transformed into a wild-type strain, selecting for the presence of the marker gene; (4) integration of the marker gene at the desired locus is then confirmed by colony PCR and the presence of the point mutation is confirmed by phenotypic screening, RFLP analysis and DNA sequencing

some cases, the mutation generates or eliminates a recognition site for an endonuclease, resulting in a restriction fragment length polymorphism (RFLP); in this case, a fragment covering the point mutation can be amplified by PCR and scored for the presence or absence of the restriction site. In other cases (c), the point mutation may affect an enzymatic activity that can be scored for. Should none of these apply (d), the presence of the desired mutation can directly be screened for by DNA sequencing. Of 10 mutations that we have successfully introduced using this procedure, nine were scorable by RFLP analysis, indicating that the generation or elimination of restriction enzyme cleavage site can frequently be associated with a given point mutation (Table 3).

Generating multiple genomic point mutations

Having confirmed the presence of the desired point mutation, an additional mutation can now been introduced by using genomic DNA from the mutant strain as a template for a PCR reaction with a primer that introduces the second mutation 5′ to the first point mutation, as outlined in Figure 2. Iteration of this procedure will thus allow the introduction of additional mutations.

As a proof of principle, we generated a mutant allele of the proton pumping plasma membrane ATPase, Pma1p. This mutation, *pma1-7*, was generated by *in vitro* mutagenesis and plasmid shuffling (Chang and Fink, 1995). Cells carrying the *pma1-7* allele display a temperature-sensitive growth phenotype and Pma1-7p affects the stability and membrane raft association of the mutant protein (Bagnat *et al*., 2001; Chang and Fink, 1995). *pma1-7* bears two point mutations, a Pro → Ala substitution in the catalytic domain at position 434 and a Gly → Ser exchange at position 789 in the cytoplasmic loop between transmembrane

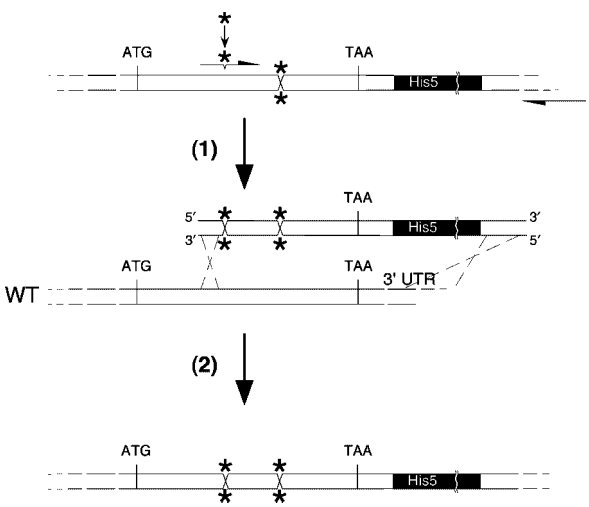

Figure 2. Strategy to generate multiple mutations. Additional point mutations can be introduced by reiteration of the basic mutagenesis scheme. Starting from the first point mutation, which must lay closest to the 3′ UTR, a second mutation is introduced by PCR amplification of genomic DNA isolated from this mutant and a primer carrying the second point mutation. Transformation of the resulting DNA fragment into a wild-type strain will then generate the desired double mutant allele

Table 3. Overview of point mutations that were generated

Name of the cassette	Cassette size (kb)	Transformation rate (col/μg DNA)	RFLP cleavage	Positive/ tested	Positive mutation/ total sequenced
PMA1::HIS5	1.48	2.5	—	2/6	—
PMA1P434A::HIS5	3.03	910	HpyCH4V	3/6	1/1
PMA1G789S::HIS5	1.91	205	TspRI	2/12	1/2
PMA1–7::HIS5	3.03	4.2	HpyCH4V/TspRI	3/8	3/3
PMA1K115R::HIS5	4.00	376	MseI	1/24	1/1
PMA1K781R::HIS5	2.00	19.2	StyI	1/10	1/1
SLC1::HIS5	1.48	22.5	—	5/10	—
SLC1Q44L::HIS5	2.38	1206	DdeI	6/30	2/6
YGR263::myc–HIS5	2.25	20	—	1/6	—
YGR263D328A::myc–HIS5	2.75	102	HhaI	2/12	1/2
YGR263S250A::myc–HIS5	3.05	259	HinfI	5/12	1/2
YGR263Y355H::myc–HIS5	2.68	1150	NlaIII	6/12	1/1
YGR263Y355F::myc–HIS5	2.68	1965	—	—	2/4*

Indicated is the gene name with the amino acid that was exchanged, the size of the PCR cassette that was used to transform wild-type cells, the generation of a novel restriction site, the number of colonies that were positive by either RFPL or PCR analysis relative to the total number of colonies that were tested, and the number of colonies that carried the desired point mutation compared to the number of colonies that were sequenced. * Determined by direct sequencing of candidate colonies.

segments 8 and 9 of Pma1p. These amino acid exchanges correspond to a C → G transversion at position 1300 of the *PMA1* ORF and a G → A transition at position 2365, respectively. We thus first tagged the wild-type *PMA1* gene with the *His3MX6* selectable marker at the 3′ UTR (Longtine *et al*., 1998). Genomic DNA from the resulting strain (RSY2331) was then isolated and used in a PCR reaction with primer Pma1G/A2365, which bears the desired G → A 2365 transition and a downstream primer (R1′Pma1) that lies 3′ to the selectable marker. The resulting fragment was then transformed into BY4741 haploid wild-type strain. The presence of the mutation was confirmed by colony PCR and DNA sequencing, resulting in a strain carrying a pma1G789S mutation (YRS2783). One of 12 tested transformants carried the desired mutation, indicating that the procedure is reasonably efficient.

To introduce the second point mutation 5′ to the first, genomic DNA from strain (YRS2783) was employed as template for a PCR reaction with a primer that now introduces the second mutation (Pma1C/G1300). The fragment was again transformed into a BY4742 haploid wild-type strain, and the presence of the marker gene was scored for by colony PCR. Positive colonies were then tested for the presence of the two point mutations by DNA sequencing, resulting in strain YRS2779. One of four colonies tested carried the desired mutation, again indicating that fixation of the point mutation is efficient.

This *ab initio* generated allele of *PMA1* showed the phenotypic characteristics of the original *pma1-7* allele (Chang and Fink, 1995). Mutant cells were temperature-sensitive for growth at 37 °C, and the mutant protein was unstable when synthesized at 37 °C, as revealed by pulse-chase analysis (Figure 3).

It should be noted that the presence of a point mutation, particularly in an essential gene, may confer a growth disadvantage or may even result in lethality, making it difficult or even impossible to isolate the desired mutation in a haploid strain background. This problem can be overcome by using either a haploid strain that carries an additional copy of the gene of interest on a plasmid with a counter-selectable marker, or by using a diploid strain to construct the desired allele. Counter-selection against the plasmid or sporulation of the diploid will then result in a haploid strain with the desired mutation.

Although the point mutation in this method is inevitably close to the 5′ end of the fragment to be inserted, the efficacy of genomic fixation of the mutation is high enough to render the approach practical, which is consistent with reports showing that the introduction of point mutation is efficient (average 24%) even when the point mutation is close (within 25–50 bp) to the 5′ of the

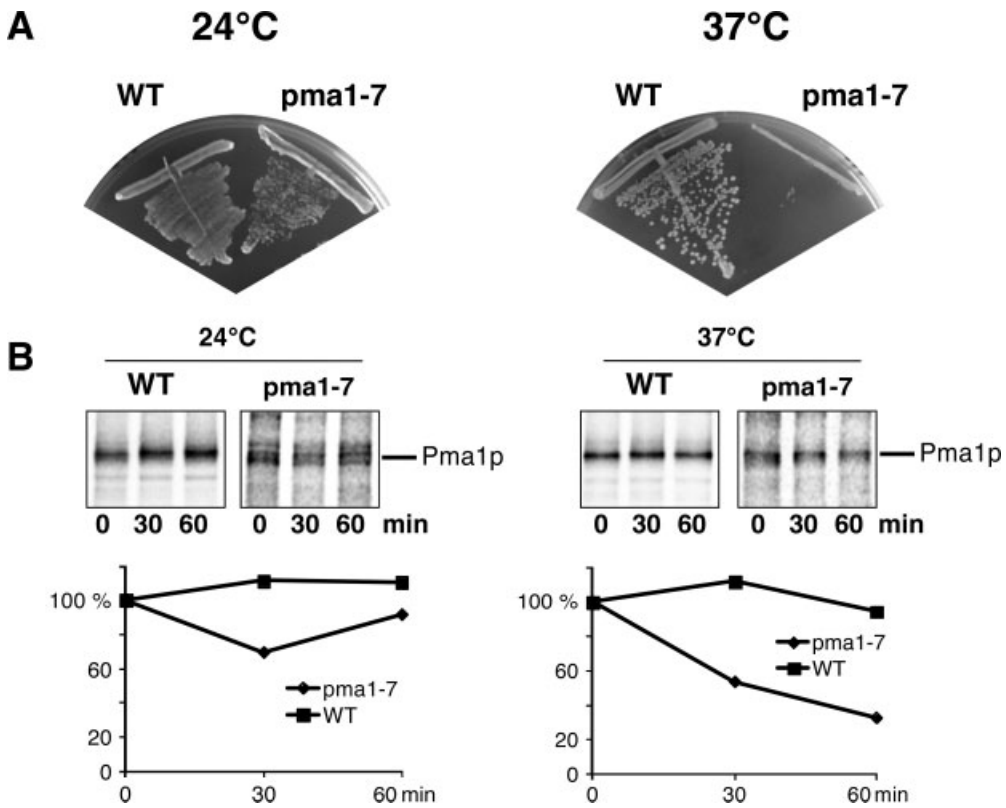

Figure 3. Generation of a temperature-sensitive allele of the plasma membrane ATPase, *pma1-7*. A strain bearing the *pma1-7* mutant allele was generated as described in the text and phenotypically characterized. (A) *pma1-7* is temperature-sensitive for growth. Wild-type (YRS1533) and *pma1-7* mutant (YRS2779) strains were plated on minimal media and incubated at either 24 °C or 37 °C. (B) Newly synthesized Pma1-7p is unstable. Wild-type and *pma1-7* mutant strains were cultivated in minimal media lacking methionine, cells were split and pre-incubated at either 24 °C or 37 °C for 15 min, pulse-labelled by the addition of [35]S-methionine and [35]S-cysteine for 5 min and then chased by the addition of unlabelled amino acids. Samples were taken at the time points indicated and Pma1p was immunoprecipitated and resolved by SDS–PAGE. Quantification of Pma1p levels is shown in the graph

homologous region that targets integration (Erdeniz *et al*., 1997).

To circumvent a possible reduction of steady-state levels of transcripts due to the insertion of the selectable marker in the 3′ UTR of the gene, as has recently been used to generate hypomorphic alleles of essential genes (DAmP allele; Schuldiner *et al*., 2005), *loxP*-flanked selectable marker genes could be used for marker targeting, in which case the marker could be recycled after successful creation of the desired point mutation, leaving the 3′ UTR intact (Gueldener *et al*., 2002).

The procedure described here can easily be employed to generate single or multiple defined mutations in the genome of *S. cerevisiae*, and thus facilitates structure–function analysis of proteins *in vivo*.

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