Two-step method for constructing unmarked insertions, deletions and allele substitutions in the yeast genome

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Abstract

We describe three extensions of the method of site-specific genomic (SSG) mutagenesis. These three extensions of SSG mutagenesis were used to generate precise insertion, deletion, and allele substitution mutations in the genome of the budding yeast, Saccharomyces cerevisiae. These mutations are termed precise because no attached sequences (e.g., marker genes or recombination sites) are retained once the method is complete. Because the method is PCR-based, neither DNA cloning nor synthesis of long oligonucleotides is required. We demonstrated the efficacy of these methods by deleting an ORF, inserting the tandem affinity purification (TAP) tag, and replacing a wild-type allele with a mutant allele.

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1. Introduction

In many organisms, it is possible to target mutations to specific sites in the genome. Targeting mutations to the genome allows the effect of specific mutations to be studied in their native genomic context. In particular, native chromosomal DNA may differ from DNA carried on a plasmid with regard to chromatin structure, flanking sequence, and copy number. Each of these features influences the function of chromosomal elements such as promoters, telomeres and replication origins. Thus, targeting mutations directly into the genome has been a valuable tool in many branches of molecular biology.

Several methods for targeted genomic mutagenesis have been developed in the budding yeast, Saccharomyces cerevisiae. Targeted mutagenesis is particularly efficient in this yeast because of the high frequency of recombination between chromosomes and exogenous DNA, termed disruption fragments (reviewed in [1]). A disruption fragment can be readily introduced into yeast by transformation [2], and when the ends of this fragment are homologous to genomic sequence, the fragment can integrate into the genome via homologous recombination. Because integration does not require homology in the internal region, targeted mutagenesis can be used to delete and/or insert sequence, depending on the sequence at the ends. Usually the internal region of the disruption fragment contains a “marker” gene, which allows selection for cells that integrate that fragment. For example, a recipient strain containing the ura3 allele fails to grow in the absence of uracil, but transformants that incorporate a disruption fragment containing the URA3 marker can be selected on medium lacking uracil.

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Targeted mutations are termed “precise” if the marker gene and other exogenous sequences can be removed, leaving only the mutation. Precise targeted mutagenesis has two advantages. First, it restores the auxotrophy, which can then be used again for a further round of targeted mutagenesis. Second, precise mutagenesis prevents disruption of the targeted region. For example, when an epitope tag is integrated into the N-terminus of an ORF, the marker gene must be removed or it will disrupt transcription from the ORF.

The most common method of precise mutagenesis requires integrating an engineered URA3 allele that can be subsequently removed. This removal can be selected using the drug 5-fluoroorotic acid (5-FOA), since Ura+ but not Ura− isolates are sensitive to this drug [3]. One type of engineered URA3 allele used for this purpose is flanked by direct repeats; homologous recombination between these repeats will “pop-out” the intervening marker [4]. One drawback to this type of engineered URA3 allele is that direct repeats can interfere with the PCR. In order to generate a disruption fragment with a removable URA3 allele completely by PCR, a template containing URA3 allele flanked by E. coli cre-lox recombination sites can be employed [5,6]. In this case, expression of the corresponding site-specific recombinase efficiently excises the marker, however the short recombination site remains, meaning that the mutation is not entirely precise.

We recently described a new method for targeting precise point mutations that does not require engineered URA3 alleles and uses PCR fragments rather than cloned genes [7]. This method, termed site-specific genomic (SSG) mutagenesis, involves two steps, and was inspired by an oligonucleotide-based method of precise mutagenesis, delitto perfetto [8]. In the first step of SSG mutagenesis, a PCR fragment is used to insert URA3 near the target site using standard methods (reviewed in [9]). In the second step, a PCR fragment homologous to the region spanning the URA3 insertion is used to replace URA3. This PCR fragment contains a point mutation (or several mutations) at its end. This mutation is present in the PCR fragment because it is contained on one of the primers used to generate the fragment. Thus, at the same time that URA3 marker is replaced, the mutation is introduced into the genome. One advantage of SSG mutagenesis over delitto perfetto is that SSG mutagenesis requires only short oligonucleotides; whereas, delitto perfetto requires long oligonucleotides (80–100 bp), which may sometimes contain unwanted mutations. Furthermore, the region targeted by SSG mutagenesis extends at least 1–2 kb from the marker; whereas, in delitto perfetto this range is only 80–100 bp.

In the present report, we describe several expanded uses of SSG mutagenesis. These methods create precise insertions, deletions and allele substitutions. To demonstrate the first method, insertion SSG (iSSG), a tandem-affinity protein (TAP) tag was inserted at the N-terminus of a protein [10]. To demonstrate the second method, deletion SSG (dSSG), an ORF was deleted. To demonstrate the third method, allele shuffling (aSSG), a mutant allele from a donor strain was substituted for the wild-type allele in a recipient strain. These methods of precise mutagenesis are rapid and simple, and they have the advantage of not requiring DNA cloning or the synthesis of long oligonucleotides.

2. Materials and methods

2.1. Yeast strains and media

All strains are in the W303 background. Insertions were performed on the strain SH2988 (MATα ade2 can1::ADE2; CAN his3-11,15 leu2-3,112 ura3-1). Deletions were performed on the strain SH773 (MATα ade2 can1::ADE2; CAN1 his3-11,15 leu2-3,112 trp1-3Δ ura3-1). Allele shuffling was performed in the strain SH2867 (MATα ade2 can1::ADE2; CAN1 his3-11,15 leu2-3,112 trp1 ura3-1 rme1 Δ:LEU2 ime1 Δ-lacZ::LEU2). All medium were prepared as described previously [11].

2.2. PCR amplification and mutation analysis

PCRs were performed as described previously [12]. PCR fragments were purified by Qiiaquick columns (Qiagen) prior to transformation or sequencing.

PCR fragments used in Step 1 contained URA3. These fragments were amplified from pRS306 [13] using 60 nt. primers that contained 40 nt. at the 5′ end homologous to the targeted region and 20 nt. at the 3′ end homologous to the region flanking URA3. In the primers shown below, the 3′ end homologous to the vector are in boldface type. For iSSG, these primers were AACAGTTTTTGCGTTTCTTTATACTAAGAGGCTATATAACTATCGGCTACAGGC and GCCTATTCGTGATGGTTTATTATCTCTGATTTCAATCCATCTGATGCGGTATTTC. For dSSG, the primers were CGCAAGGGCTTTGTTAATGGCGGTGACATGTGTTCTAGTTAATCAGATCGGCTACAGGC and ATCCCCCTCGGGTCATTGATGAAGATTCCAGGAGCATTATCCTGATGATTTATCTCC. For aSSG, the primers used to construct SH2982 have been described [7].

For step 2 of iSSG, a PCR fragment containing the TAG tag was amplified from pBS1761 [14] using the primers CAGTTTTTGCGTTTCTTTATACTAAGGCTATATAACTATCGGCTACAGGC and GCCTATTCGTGATGGTTTATTATCTCTGATTTCAATCCATCTGATGCGGTATTTC. For step 2 of dSSG, a PCR fragment was amplified from wild-type genomic DNA using the primers CGCC-
AAGGGCTTGTTAATGCGGTACATGTTCTTAGCTATCTGCTCTGAATCTTC and TGAGGGAGGATTGGAACCT. For step 2 of asSSG, a PCR fragment was amplified from genomic DNA containing the ime1-sff mutant allele (SH2785) using the primers GTACCCTACAGCTATCTGTCTTAG and TTTCAACAGTAATGGGTGCCCAGTAG.

Diagnostic PCRs performed on microwaved cells were used to identify transformants containing insertions, deletions or point mutations [7]. To detect insertions and deletions, primers flanked the targeted region. For iSSG, the primers used were pr1 (GGGAAGCCCAA-AATTAAGG) and pr2 (GAATTCTGGATTTGCTTCC). For dSSG, the flanking primers were pr3 (GCAAAGAAAGGCTTGAGAG) and pr4 (TGAGGGAGGATTGGAACCT) and the diagnostic PCR of all strains also contained a third primer, pr5 (CCGGTGTGGGTTTAGATGAC) homologous to URA3. The third primer was necessary because URA3 is approximately the same size as the deleted region. For asSSG, it was necessary to detect the presence of point mutations, so two parallel diagnostic PCRs using the same 3′ primer pr7 (GCTCC CTTTAAAATGCAC) were performed. In the first PCR, the final nucleotide of the 5′ primer corresponded to one of two mutations (pr8, AAGTGGTATCCTTTGTGAA, mutations shown in bold). In the second PCR, the 5′ primer corresponded to the wild-type sequence (pr7, AAGTGGTATCCTTTGTTAC).

2.3. Transformations

Step one and step two transformations contained approximately 1 μg of PCR fragment and employed a high-efficiency lithium acetate transformation method [2]. Ura + transformants generated in Step 1 were screened by diagnostic PCR, and at least one isolate containing the correct insertion was then transformed in Step 2. As described previously, in order to select for loss of URA3 in step two, it was necessary to cotransform the PCR fragment with a plasmid containing a second marker gene [7]. In this case, we used 0.3 μg of a 2-μm plasmid containing TRP1 (pTV3), and the transformants were plated on Trp - medium. After 60 h, Trp + colonies were replica-plated to 5-FOA medium. FOA R colonies appearing after 24–48 h were screened by diagnostic PCR.

3. Results and discussion

3.1. Precise insertions into the genome

One of the most important tools in molecular genetics is the fusion gene, in which the ORF of a gene of interest is fused in frame with either a reporter gene (to measure gene expression) or a tag (to identify or purify the gene product). In order for expression of the fusion allele to accurately reflect expression of the wild-type allele, fusion genes can be constructed at the native genomic locus in budding yeast [15–17]. Because the tag may interfere with the function or stability of the targeted gene, it is useful to construct two alternative fusion genes, one fused at the C-terminus and one fused at the N-terminus. However, when the tag is fused at the N-terminus, marker genes and other intervening sequences must be removed or they will interfere with transcription. Construction of an N-terminal fusion in which intervening sequences were removed (i.e., a precise insertion) was performed using a version of SSG mutagenesis termed insertion SSG (iSSG) mutagenesis. As in the original SSG mutagenesis protocol [7], iSSG mutagenesis involves two sequential steps (Fig. 1A).

In the first step, a PCR fragment containing URA3 is inserted into the target region without deleting genomic sequences, and transformants are selected on medium lacking uracil (Ura - medium). In the second step, one of the isolates generated in the first step is transformed with a second PCR fragment, which lacks a marker gene but which contains the insertion sequence. Transformants in which URA3 is replaced with the inserted sequence are selected on medium containing 5-FOA.

iSSG mutagenesis was used to create a precise genomic fusion between the tandem affinity purification (TAP) tag [10,14] and the GRR1 ORF. In the first step, yeast were transformed with a PCR fragment containing URA3 flanked at either end by 40 bp that was homologous to either side of the N-terminus of the ORF (Fig. 1A) to generate an intermediate strain. In the second step, this intermediate strain was transformed with a PCR fragment that contained the TAP tag and the same 40 bp tails as in the first step. Transformants from both the first and second steps were identified by diagnostic PCR (Fig. 2A and 2D lanes 2–4). Diagnostic PCR was performed using primers flanking the insertion site. In the first step, among 28 Ura + transformants, four contained URA3 inserted at the correct site. As demonstrated previously in this strain background [12], the remaining Ura + transformants result from conversion of the ura3-1 allele. This conversion may possibly be reduced by substituting the S. cerevisiae URA3 with the partially homologous URA3 gene from K. lactis [18]. In the second step, 10 FOAR isolates were identified, of which five contained a precise insertion of TAP. The remaining five FOAR isolates likely resulted from gene conversion of URA3 to ura3-1. For two isolates containing a perfect insertion, we verified that the insertion was precise and that no other mutations were introduced by amplifying the region of the insertion by PCR as described [7] and sequencing. In these two isolates and a control strain lacking the tagged gene, protein was extracted, precipitated with calmodulin–sepharose
and analyzed by Western blot as described Puig, 2001 #1437. The Western blot revealed a single band corresponding to TAP-Grr1p in both tagged isolates that was absent in the control strain (Fig. 2E).

The frequency of FOA^R isolates that contained a deletion of *URA3* (50%) was comparable to this frequency in the standard SSG method (60–100%) [7]. We also used iSSG to create a precise N-terminal TAP tag at a second gene, *FKH2* (not shown). iSSG has the advantage over previous methods of precise insertion mutagenesis because the tag can be precisely inserted into the genome without expression of exogenous recombinases [19,20]. However, because tandem repeats of short epitope tags (e.g., 3XHA or 12XMYC) may not amplify efficiently, precise insertion of these tags may be better accomplished by other methods [16].

### 3.2. Precise deletions of genomic DNA

Deletions of specific genomic regions were created by a version of SSG mutagenesis termed deletion SSG (dSSG) mutagenesis (Fig. 1B). In the first step, the targeted region is replaced with *URA3*, and transformants are selected on Ura^- medium. In the second step, *URA3* is deleted from the genome, and transformants selected on 5-FOA medium.

We used dSSG to precisely delete the *FKH1* ORF. In the first step, yeast was transformed with a PCR fragment which contained *URA3* flanked at either end with 40 bp of sequence corresponding to either end of the *FKH1* ORF [21]. In the second step, the strain generated in step 1 was transformed with a PCR fragment that contained 40 bp flanking one side of the *URA3* insertion and 600bp homology flanking the other side. As a result, *URA3* was precisely deleted in the second step.

Transformants from both steps of dSSG were screened by diagnostic PCR using primers flanking the target. For the first step, 3 out of 38 Ura^+ transformants correctly replaced *FKH1* with *URA3*. For the second step, 19 FOA^R colonies, 3 were shown to have precisely deleted the *FKH1* gene (Fig. 2B and 2D, lanes 5–7). To confirm the general applicability of dSSG, we successfully used dSSG to precisely delete a 51 aa domain from an ORF (not shown). One advantage of dSSG over existing methods is that dSSG can be performed on any existing *URA3*-marked deletion mutant, whereas other methods require first making the deletion

![Fig. 1. Strategy for generating precise insertions (iSSG), deletions (dSSG), and allele substitutions (asSSG).](https://academic.oup.com/femsle/article-abstract/248/1/31/556371)
using an engineered URA3 and subsequently removing this marker.

3.3. Allele shuffling by SSG mutagenesis

Transfer of particular alleles from one strain to another was accomplished by allele-shuffling SSG (asSSG) mutagenesis (Fig. 1C). In the first step, a target gene is replaced by URA3, and transformants are selected on medium lacking uracil to generate a recipient strain. In the second step, URA3 in the recipient strain is replaced by a mutant allele of the target gene, and transformants are selected on 5-FOA medium and screened by diagnostic PCR.

We employed asSSG to transfer an allele of IME1 from a donor to a recipient strain. In the first step, we replaced a 750 bp region of the IME1 promoter with URA3 as in dSSG. In the second step, we transformed this recipient strain with a 1.5 kb fragment amplified from a donor strain. This fragment contained the 750 bp region that was deleted in the recipient strain, 200–600 bp flanking either side of this region and a point mutation present within the 750 bp region in the donor strain.

Incorporation of the mutation into the recipient genome was verified by two parallel diagnostic PCRs (Fig. 2C and 2D). These two PCRs used the same 3' primer and different 5' primers. These 5' primers differed only in their final 3' nucleotide. In one PCR, this nucleotide corresponded to a mutation; whereas in the other PCR, it corresponded to the wild-type sequence. Of 30 FOAR colonies screened, 8 contained the mutation (27%). Of these isolates, three were selected, and the targeted region was amplified and sequenced in these three isolates. For each of these isolates, we found that the targeted mutation was present and that the remaining sequence was wild-type. Thus asSSG is an effective means of transferring alleles from one strain to another. Note that the same strain can be used as the recipient for many donor alleles.

3.4. Advantages of iSSG, dSSG, and asSSG methods for precise genomic mutagenesis

Most methods for targeting mutations into the yeast genome introduce a selectable marker gene together with the mutation. This marker must be removed in order to create precise mutations. As described above, precise insertions, deletions, and allele substitutions can be generated using PCR fragments, rendering DNA cloning and long oligonucleotides unnecessary. The simplicity and efficiency of these methods suggests that they may be applicable in other microorganisms that display high frequencies of recombination directed by short regions of homology.

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