



Functional Analysis Reports

Precise Gene Disruption in *Saccharomyces cerevisiae* by Double Fusion Polymerase Chain Reaction

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We adapted a fusion polymerase chain reaction (PCR) strategy to synthesize gene disruption alleles of any sequenced yeast gene of interest. The first step of the construction is to amplify sequences flanking the reading frame we want to disrupt and to amplify the selectable marker sequence. Then we fuse the upstream fragment to the marker sequence by fusion PCR, isolate this product and fuse it to the downstream sequence in a second fusion PCR reaction. The final PCR product can then be transformed directly into yeast. This method is rapid, relatively inexpensive, offers the freedom to choose from among a variety of selectable markers and allows one to construct precise disruptions of any sequenced open reading frame in *Saccharomyces cerevisiae*.

KEY WORDS — gene disruption; fusion PCR; *Saccharomyces cerevisiae*

INTRODUCTION

The sequence of the entire yeast genome will soon be known and with this knowledge will come an unprecedented opportunity to advance our understanding of the genes required to construct a eukaryotic organism. One of the best and most frequently employed tools for understanding gene function in *Saccharomyces cerevisiae* is the construction of gene disruptions by replacement of the gene of interest with sequences coding for selectable markers (Rothstein, 1983). This procedure is facilitated by efficient homologous recombination in this yeast. In the past this has been done by using restriction enzymes to replace sequences of the gene of interest with a cassette carrying a marker gene, both carried on suitable vectors. This method can be inflexible or time consuming because it requires using the existing restriction sites, or engineering additional unique sites, for inserting the DNA fragment encoding the selectable marker.

Recently we developed an alternate method for the construction of replacement alleles which does

not require plasmid intermediates and allows the researcher to design the exact configuration of the replacement allele. This method employs a two-stage fusion polymerase chain reaction (PCR) (Ho *et al.*, 1989; Dillon and Rosen, 1990) strategy. We have used this method to disrupt several genes in yeast. Here we describe its application to the disruption of a gene we call *AIP1*. *AIP1* encodes a recently identified actin interacting protein (Amberg *et al.*, 1995); however, its identity is unimportant for this method, as any yeast gene of known sequence can be disrupted by this protocol.

MATERIALS AND METHODS

Plasmids and primers

The template for the *AIP1* PCR reactions was plasmid pAIP1-1, which carries a genomic copy of the *AIP1* gene (unpublished). Plasmid DNA was prepared by use of a Qiagen 500 column according to the manufacturer's instructions (Qiagen Inc.). The templates for the marker PCR reactions were a series of vectors that contain yeast selectable markers in pUC18: pJJ215 (bearing the *HIS3*

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Table 1. *Saccharomyces cerevisiae* strains.

Name	Genotype
DBY6525	a/a <i>ura3-52/ura3-52 leu2Δ 1/leu2Δ1 HIS3/his3Δ200 trp1Δ63/TRP1</i>
DBY6526	a/a <i>ura3-52/ura3-52 leu2Δ1/leu2Δ1 HIS3/his3Δ200 trp1Δ63/TRP1 AIP1/aip1::URA3</i>
DBY6527	a <i>ura3-52 leu2Δ1 his3Δ200 trp1Δ63 aip1::URA3</i>
DBY6528	α <i>ura3-52 leu2Δ1 his3Δ200 trp1Δ63 aip1::URA3</i>
DBY6529	a <i>ura3-52 leu2Δ1 his3Δ200 trp1Δ63 aip1::LEU2</i>
DBY6530	a <i>ura3-52 leu2Δ1 his3Δ200 trp1Δ63 aip1::HIS3</i>
DBY6531	α <i>ura3-52 leu2Δ1 his3Δ200 trp1Δ63 aip1::TRP1</i>

gene), pJJ282 (bearing the *LEU2* gene), pJJ281 (bearing the *TRP1* gene) and pJJ242 (bearing the *URA3* gene) (Jones and Prakash, 1990). The use of these vectors allows all the marker to be amplified with an M13 'forward' sequencing primer (5'-CGCCAGGGTTTTCCAGTCACGAC-3') and an M13 'reverse' sequencing primer (5'-AGCGGAT AACAATTCACACAGGA-3').

The primers for amplification of the 5' end of *AIP1* were DAo-AIP1-1 (5'-CCACCTCAGCCTT CGACA-3') and DAo-AIP1-2a (5'-GTCGTGACT GGGAAAACCCTGGCGCATAGGTACTGGGA ACCC-3'). The primers for the amplification of the 3' end of *AIP1* were DAo-AIP1-3a (5'-TCCTGT GTGAAATTGTTATCCGCTGGACTTAGTTGC CACTGG-3') and DAo-AIP1-4 (5'-CTCACTCG AGGACAACAT-3'). Regions complementary to the M13 sequences flanking the selectable marker are shown in bold type.

Strains

Plasmids were amplified in bacterial strain DH5α. Integrative transformations were performed by electroporation (Guthrie and Fink, 1991). Strains DBY6527 and DBY6528 were derived by sporulation and dissection of strain DBY6526 (Table 1).

PCR Reactions

The initial marker and flanking sequence PCR reactions were executed in 50 μl that contained 20 mM-Tris pH 8, 10 mM-KCl, 10 mM-(NH₄)₂SO₄, 2 mM-MgSO₄, 0.1% Triton X-100, 10 ng template DNA, 500 nM of each primer, 200 μM-dNTPs, 1 μl Vent (2 units) polymerase (New England Biolabs, Inc.). The reactions were first heated to 94°C for 4 min, then 30 cycles of: 94°C for 1 min, 55°C for 1 min, 72°C for 3 min. Extensions were continued at 72°C for a further 20 min.

PCR products were purified by separation through low melting agarose (SeaPlaque from FMC Bioproducts) in 0.5TAE Buffer (Sambrook *et al.*, 1989).

The fusion PCR reactions were done in a volume of 50 μl containing 20 mM-Tris pH 8.3, 1.5 mM-MgCl₂, 25 mM-KCl, 0.05% Tween 20, 5 μl purified PCR product (low melting agarose was liquefied by incubation at 65°C), 500 nM of each primer, 200 μM-dNTPS, 100 μg/ml acetylated bovine serum albumin (New England Biolabs, Inc.), 1 μl Taq polymerase (5 units, GibcoBRL Inc.). The reaction conditions were otherwise as stated for the previous PCR reactions.

The final fusion PCR product was purified by extraction with 50% phenol/50% chloroform followed by ethanol precipitation.

Colony PCR was performed as for the fusion PCR reactions except that a small yeast colony replaced the template DNA, primers DAo-AIP6-1 and DAo-AIP6-4 were the primers used and 35 not 30 cycles of amplification were performed.

RESULTS

Construction of an *AIP1* replacement allele

The strategy for constructing replacement alleles by double fusion PCR is shown in Figure 1. The procedure requires a total of five PCR reactions: three standard PCR reactions and two fusion PCR reactions. The three standard PCR reactions are performed in step 1 (see Figure 1). These include one reaction to amplify the marker of choice, and two reactions to amplify the regions flanking the site of marker insertion in the disruption target. In step 2, the first fusion PCR reaction fuses the 'upstream' flanking region to the marker gene. In step 3, the second fusion PCR reaction fuses the 'downstream' flanking sequence to the product of

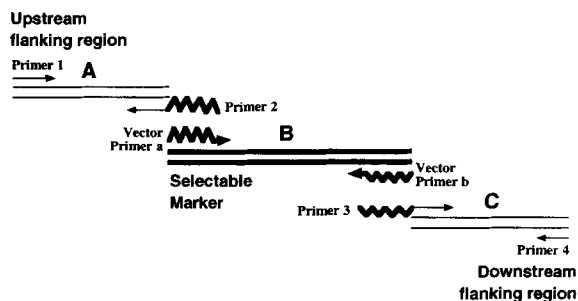
the first fusion PCR reaction. The product of the second fusion PCR reaction contains the upstream and downstream sequences of the target gene separated by a functional marker gene. Obviously, the fusion reactions could be done in the other order and the end product would be the same.

For the selectable marker templates we chose to use a set of vectors constructed by Jones and Prakash (1990). These vectors contain the four most frequently used yeast markers (*HIS3*, *LEU2*, *TRP1* and *URA3*) cloned into the polylinker sequence of pUC18 in both orientations. Marker sequences can be amplified from these templates by using the standard M13 forward and reverse sequencing primers. Most molecular biology laboratories have stocks of these primers and can therefore avoid the cost of purchasing them for this procedure. Initially we decided to make a replacement allele carrying the *URA3* gene, amplified from plasmid pJJ242 (Jones and Prakash, 1990), using the M13 primers.

The gene we first disrupted using this procedure, *AIP1*, was identified as an actin interacting protein (Amberg *et al.*, 1995) using the two-hybrid protein interaction reporter system (Chien *et al.*, 1991). At the time of these experiments, we only had the sequence of the ends of a cDNA clone encoding *AIP1* fused to the *GAL4* activation domain.

The primers used to amplify the disruption target are of two classes: the primers most distal from the insertion site for the selectable marker (Figure 1, primers 1 and 4) are simply complementary to the gene target and can be relatively short; the primers that are directly proximal to the site of insertion of the selectable marker (Figure 1, primers 2 and 3) are chimeric, containing sequences complementary to the primers used to amplify the selectable marker at their 5' end, followed by sequences complementary to the gene target. The primers used to amplify the 5' end of *AIP1* were DAo-AIP1-1, which is complementary to the antisense strand of *AIP1* at the 5' end of the cDNA sequence, and DAo-AIP1-2a, which begins with 24 nucleotides complementary to the M13 forward primer and ends with 18 nucleotides complementary to the sense strand of *AIP1*, 216 nucleotides downstream from DAo-AIP1-1. The primers used to amplify the 3' end of *AIP1* were DAo-AIP1-3a, which begins with 24 nucleotides complementary to the M13 reverse primer and ends with 18 nucleotides complementary to the antisense strand of *AIP1*, 189 nucleotides up-

STEP 1. Three primary PCR reactions



STEP 2. 1st Fusion PCR



STEP 3. 2nd Fusion PCR

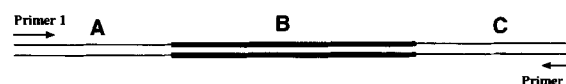


Figure 1. Strategy for constructing gene disruption cassettes by PCR. Step 1: the three primary PCR reactions. The regions flanking the site of selectable marker insertion ('A' and 'C') are amplified with oligonucleotide primers specific for the target sequence. The primers distal from the selectable marker insertion site are simple primers complementary to the target sequence (straight, fine line). The primers directly adjacent to the insertion site are chimeric: their 5' end is complementary to the primers used to amplify the selectable marker sequence (wavy, bold line) and their 3' end is complementary to the target sequence (straight, fine line). The selectable marker gene sequence ('B') is amplified with primers complementary to the vector sequences adjacent to the marker gene (wavy, bold line). In our case, the selectable marker genes are cloned into the pUC18 vector and we used M13 forward and reverse sequencing primers. The products of these three, separate PCR reactions are purified and used as templates in the next two steps. Step 2: first fusion PCR reaction. The first flanking region is joined to the selectable marker. As illustrated, products A and B are used as templates, amplified with primer 1 and vector primer b to generate a fusion product linking the upstream flanking region to the selectable marker. The order in which the flanking sequences are fused to the selectable marker is discretionary. This product is purified and used as one of the templates in the next step. Step 3: second fusion PCR reaction. The second flanking region is joined to the product from step 2. As illustrated, the A-B product from step 2 and product C from step 1 are used as templates in a PCR reaction, amplified with primers 1 and 4 to generate the final gene disruption construct (A-B-C). This product can be purified and used directly to transform yeast.

stream from the stop codon, and DAo-AIP1-4, which is complementary to the *AIP1* sense strand beginning with the stop codon.

Vent polymerase was used in the first PCR reactions because of its relatively lower error rate as compared to Taq polymerase (New England Biolabs, Inc.). The 216 bp *AIP1* upstream product, the 189 bp *AIP1* downstream product and an approximately 1200 bp product carrying the *URA3* gene were purified on a low melting agarose gel (data not shown).

The gel slices containing the upstream flanking region and *URA3* PCR products were melted and a small amount of each was added to a fusion PCR reaction containing primers DAo-AIP1-1, DAo-AIP1-3a and Taq polymerase. Taq polymerase was used because the agarose was found to inhibit Vent polymerase; however, Vent polymerase could be used if the templates for the fusion PCR were purified from the agarose. The resulting 1400 bp fusion PCR product was purified as before on a low melting agarose gel.

For the final fusion PCR reaction, the downstream PCR product and the product of the first fusion PCR reaction were mixed and the full-length fusion was amplified with primers DAo-AIP6-1 and DAo-AIP6-4 using Taq polymerase. This final 1600 bp product, with *AIP1* flanking sequences fused to *URA3*, was purified on an agarose gel, extracted with phenol then chloroform and finally ethanol precipitated.

Gene replacement in yeast

The purified double fusion PCR product was transformed into a wild-type diploid strain DBY6525 by electroporation, and transformants were selected on minimal media lacking uracil. Four independent isolates were tested for correct integration by colony PCR using primers DAo-AIP1-1 and DAo-AIP1-4 (data not shown). In all four cases both products corresponding to the wild-type allele and the replacement allele were amplified (data not shown).

One of these strains (DBY6526) was sporulated and tetrads were dissected. All four progeny from several tetrads were viable and the replacement allele was found to segregate 2:2 (data not shown). Colony PCR on several Ura⁺ progeny strains indicated only the presence of the replacement allele. One such result is shown in lane 2, Figure 2. A Western assay using an antibody specific to Aip1p confirmed the absence of the Aip1 protein in strain DBY6527 (data not shown).

The strain carrying the *URA3* replacement was a useful reagent for constructing other replacements at the *AIP1* locus. Double fusion PCR was used to

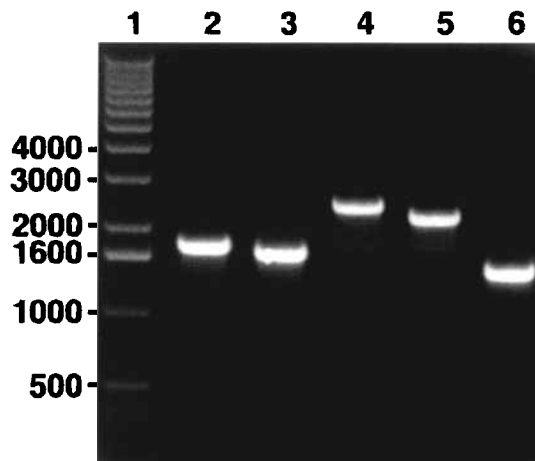


Figure 2. Colony PCR of yeast strains. Colony PCR was performed on yeast strains using primers DAo-AIP1-1 and DAo-AIP1-4. The amplified DNA was separated on an agarose gel. A photograph of the ethidium bromide-stained gel is shown. (Lane 1) DNA size markers; (lane 2) DBY6525 (wild type); (lane 3) DBY6528 (*aip1::URA3*); (lane 4) DBY6529 (*aip1::LEU2*); (lane 5) DBY6530 (*aip1::HIS3*); (lane 6) DBY6531 (*aip1::TRP1*).

construct *HIS3*, *LEU2* and *TRP1* replacement alleles in the same manner as the initial *URA3* replacement allele was constructed, the DNA was purified and transformed into strains DBY6527 or DBY6528, both derived from sporulation of DBY6526 and both of which contain *URA3* replacements at the *AIP1* locus. Transformants were selected on media lacking histidine, leucine or tryptophan and then transformants that had lost the *URA3* marker were identified by growth on medium containing 5-fluoro-ortotic acid. The new replacements were confirmed by colony PCR (Figure 2) and in all cases only the product of the expected size was detected. The choice of the Jones and Prakash vector set allowed the same set of six primers, four of them unique to the *AIP1* sequence, to be used for all of these constructions.

DISCUSSION

One of the first questions a yeast geneticist asks about any newly identified gene, is whether a null allele of that gene causes any aberrant phenotypes that may help to illuminate the gene's function. It is common, and relatively trivial, to construct such null alleles in yeast. Usually this procedure is done by replacing all or part of the gene's reading frame with a selectable marker, excising this allele from

the vector in which it was constructed and transforming this linear fragment into a wild-type diploid yeast strain. The resulting hemizygote is then sporulated and the progeny spores bearing the null allele are analysed. This process requires intermediate subcloning steps and the exact configuration of the replacement allele is limited by the location of useful restriction sites. Often one must choose a less than optimal configuration, either leaving some of the reading frame intact or removing flanking sequences that may affect the expression of important neighboring genes. Recently a one-step PCR procedure was described for gene disruption in yeast (Baudin *et al.*, 1993; Wach *et al.*, 1994). Although this protocol avoids the problems associated with having to construct the replacement allele by subcloning, it can suffer from poor efficiency of homologous integration. This is because the homologous sequences used to direct integration are encoded in the primers and are therefore of a limited length, usually only 50 bases.

We have developed a two-stage fusion PCR strategy that allows the researcher to design the exact configuration of the replacement allele, including the length of the flanking homologous sequences, used to direct integration. This procedure is rapid and requires no intermediate subcloning steps. Sufficient quantities of DNA encoding the replacement allele are produced by the final fusion PCR reaction such that it can be directly transformed into yeast. All one requires is at least partial sequence for the gene of interest to design the four gene-specific primers and two primers for amplifying the selectable marker. Two of these gene-specific primers are useful for confirmation of integration of the replacement allele and can be used to follow the replacement alleles in subsequent crosses, by colony PCR. The procedure does not require a plasmid clone of the gene of interest, as yeast genomic DNA can be used as the template for the primary PCR reactions of the upstream and downstream flanking sequences.

We have chosen to use the Jones and Prakash (1990) vectors as the templates for the marker PCRs because of the flexibility they provide. These plasmids are readily available from the ATCC. With the same six primers and these eight vectors as templates, eight different replacement alleles can be constructed. One has the choice of four different selectable markers (*HIS3*, *LEU2*, *TRP1* and *URA3*) and transcription in either direction. In some cases transcriptional interference can affect

the ability of the markers to complement a strain's auxotrophy. In these cases it would be important to try building the replacement allele with the marker transcribed in the opposite orientation.

In this study, we first constructed a *URA3* replacement allele, then used this strain for integration of the other alleles. First we selected for the marker of interest and then tested for loss of the *URA3* gene by ability to grow on 5-fluoro-oroic acid. In this manner, we were able to confirm that integration had occurred at the expected location without repeating the more arduous methods that were used to confirm the first *URA3* replacement.

We believe this procedure will be very useful to yeast geneticists, particularly as more, and soon all, of the yeast genome is sequenced. However, we hope that researchers working with other model organisms will recognize the general usefulness of this method and attempt similar gene disruptions in their favorite organisms.

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