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PCR-mediated direct gene disruption in *Schizosaccharomyces pombe*

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ABSTRACT

We have examined the feasibility and efficiency of PCR-mediated direct gene disruptions in the fission yeast *Schizosaccharomyces pombe*. In the present study, the *S.pombe ura4*$^+$ gene was amplified by PCR with oligonucleotides that had short flanking regions (~40 bp) to the target gene. Using this purified PCR product we were able to disrupt genes in an *S.pombe* strain bearing a *ura4* deletion, with an efficiency ranging between 1 and 3% among selected transformants. The results indicated that despite *S.pombe*'s preference for non-homologous or illegitimate recombination, even very short stretches of homologous regions could be used to target genes at a defined frequency in this organism. The successful disruption of four independent genes (*sts1*$^+$, *gcs1*$^+$, *gsh2*$^+$ and *hmt1*$^+$) by this method further demonstrates that, despite the relatively low efficiency, the method is very feasible, and it's simplicity, especially when coupled to phenotype-based screening, should greatly facilitate disruption of genes in *S.pombe*.

The ability to carry out targeted gene disruptions by homologous recombination is one of the major means for functional analysis of different genes in yeast. The need for rapid and efficient strategies for carrying out such gene disruption have grown with the progress, and now the completion of the *Saccharomyces cerevisiae* genome sequencing project. Different disruption strategies and methods have recently been described for use in *S.cerevisiae*. The simplest method for gene disruption in this yeast uses a PCR-mediated gene disruption strategy where the selection marker is amplified by PCR with oligonucleotides that have ~35–50 bp flanking region on either side. This short flanking region on either side of the selection marker gene has been shown to provide sufficient length of homology to permit a high frequency of gene disruptants at the targeted locus upon transformation in *S.cerevisiae* (1–4).

The fission yeast *Schizosaccharomyces pombe* is also being intensely studied as a model eukaryote, and indeed, in many features it appears to have a closer relationship to higher eukaryotes than *S.cerevisiae*. A major drawback to the functional analysis of genes in *S.pombe*, however, is the relative difficulty of gene disruption through homologous recombination in this organism. This deficiency is being even more acutely felt with the rapid release of sequences from the Project for the sequencing of the *S.pombe* genome at the Sanger Sequencing Centre, UK. Although gene disruptions by homologous recombination in this yeast can be carried out, the requirement for longer regions of homology, and the apparent preference for illegitimate or non-homologous recombination in this yeast have been a major deterrent to the functional analysis of genes by use of the rapid and simple methods available for *S.cerevisiae* (5,6). Gene disruption in *S.pombe* therefore has invariably required multiple cloning steps. We have sought to determine whether the simple PCR disruption methods in use in *S.cerevisiae* were at all feasible in *S.pombe* and, if so, with what kind of efficiency. In this report we describe that, contrary to expectations, the disruption of genes in *S.pombe* with flanking regions of ~40 bp of the target gene are indeed possible. Furthermore, the frequency of correct disruptions with such short flanking regions range between 1 and 3% among selected transformants.

We initially carried out our investigations with the *S.pombe sts1*$^+$ gene (7). This gene, which is the *S.pombe* homologue of the *S.cerevisiae ERG4* gene, encodes the last enzyme in ergosterol biosynthesis (8). Disruption of *sts1*$^+$ has been shown to lead to an increased drug-sensitivity and calcium ion-sensitivity (7). Oligonucleotides for carrying out the PCR-mediated disruption of this gene were designed that could amplify the *S.pombe ura4*$^+$ gene from a *ura4*$^+$ containing plasmid with ~40 bp of flanking region on either side that had homology to *sts1*$^+$. These oligonucleotides were STSDEL1, 5′-ATGGCTTATGAAAAGTTTGGTTTTATGCTTATCTTTTGGCTATATGTATGCAT-3′ and STSDEL2, 5′-AATCAGCAGTATAATGAATTTTACGAGCGTACCTATACCAACACCAATGTTTATAACC-3′. The underlined regions correspond to *ura4*$^+$ sequences, and the remaining sequences are the flanking residues; 39 bp flanking residues in the case of STSDEL1 and 42 bp in the case of STSDEL2. The template DNA was plasmid pBSK-*ura4*$^+$, a pBSK plasmid containing the *S.pombe ura4*$^+$ gene cloned into the *Hin*dIII site of pBSK. The PCR reaction was carried out using a mixture of *Taq* and vent polymerases. After amplification by PCR, the PCR product was purified by silica particles (9) and ~0.5–1.0 µg of DNA was used to transform an *S.pombe ura4-Δ18* strain that carried a complete deletion in the *ura4*$^+$ gene (10). (A *ura4Δ-18* strain that carried a complete disruption in the *ura4*$^+$ locus has been used in all these experiments so as to minimize recombinations at the *ura4*$^+$ locus.) Transformation was carried out by the lithium acetate

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method exactly as described earlier (11), and transformants were selected on minimal medium containing adenine but lacking uracil. Transformants obtained were patched onto YES (Yeast Extract + Supplements) medium and then replica plated onto minimal medium plus adenine but lacking uracil (to record the stable Ura$^+$ colonies), as well as on YES medium containing either cycloheximide, crystal violet or calcium chloride. Approximately 45% of the colonies were stable Ura$^+$ colonies. Of these, colonies that showed sensitivity to all three media were picked up as putative *sts1* disruptants. These were confirmed by PCR (data not shown). The combined data from several independent experiments is shown in Table 1. An overall frequency of ~2% was obtained indicating that *S.pombe* genes could be disrupted by this PCR-mediated strategy. To examine if this frequency extended to other unlinked genes, we carried out disruptions of *gcs1*$^+$ (γ-glutamyl cysteine synthetase) located on chromosome I (cosmid c22F3), *gsh2*$^+$ (glutathione synthase) located on chromosome I (cosmid c3F10) and *hmt1*$^+$ (vacuolar heavy metal transporter) located on chromosome III, using similarly designed oligonucleotides that also had short flanking residues to the target gene that have been described elsewhere (11). The oligonucleotides designed for the *gcs1*$^+$ deletion had 40 and 42 bp flanking residues, the oligonucleotides for the *gsh2*$^+$ deletion had 42 and 41 bp flanking residues, while those for the *hmt1*$^+$ deletion had 42 and 40 bp flanking residues. We were able to obtain successful disruptions of all these genes using the PCR products obtained with these oligonucleotides, and the frequencies at which they were obtained are shown in Table 1. The disruptants were initially screened by their phenotypes. *gcs1* and *gsh2* disruptants are both glutathione auxotrophs and cadmium-sensitive (11) while *hmt1* disruptants are cadmium-sensitive (12). The putative disruptants were picked up and disruptions at the target locus were confirmed by PCR (data not shown). The frequencies obtained in the case of these genes also lay between 1 and 3%, and reveal that, despite the propensity of *S.pombe* to carry out illegitimate recombination (13,14), PCR-mediated gene disruption with short flanking regions is indeed feasible in *S.pombe*. To examine whether in the targeted disruptants we were also observing unlinked integrations in addition to the target site, we examined the targeted disruptions for the possibility of multiple integrations by Southern blotting using the *ura4*$^+$ gene as a probe. Among the disruptants tested (two *sts1*Δ, three *gcs1*Δ, one *hmt1*Δ and one *gsh2*Δ) we were unable to observe a second unlinked integration in any of the seven cases (data not shown). Our results indicate that although the frequency of gene disruptions seen in *S.cerevisiae* with similar flanking regions and with equivalent strains carrying deletions in the selection markers is much higher (>50%) (3), the 1–3% frequency that we have observed in *S.pombe* still makes this method a very workable one, as we have demonstrated through successful disruption of four independent genes. This observation should encourage *S.pombe* researchers to carry out gene disruptions of their target genes of interest using the PCR-mediated strategy. The method could be coupled with either phenotype-based screening (wherever the phenotype is either known or suspected) or alternatively through PCR screening by using pools of putative disruptants. While phenotype-screening makes the method almost as convenient as it is for *S.cerevisiae*, we believe that even screening by PCR of pooled samples would be generally preferable to multiple cloning steps that are currently being used for gene disruption in *S.pombe*.

Table 1. Frequency of gene disruption with short flanking regions in *S.pombe*

Target gene	No. of stable Ura$^+$ colonies	Colonies with disruption at target locus	% Frequency
sts1$^+$	301	5	2
gcs1$^+$	90	3	3
gsh2$^+$	94	1	1
hmt1$^+$	61	1	2

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