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arg3+, a new selection marker system for *Schizosaccharomyces pombe*: application of *ura4+* as a removable integration marker

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The yeast *Schizosaccharomyces pombe* is becoming increasingly popular as a genetically amenable model system for the study of diverse cellular processes from signal transduction pathways and cell-cycle control to genome organisation and recombination. A vast array of mutant strains has been created (1,2), encompassing many of these cellular functions, and mutagenesis procedures are well documented (3,4).

The development of efficient transformation procedures for *S.pombe* (5) has allowed the cloning of wild-type fission yeast genes and functionally homologous genes from other organisms by *trans*-complementation of a mutant phenotype.

Ectopic expression of genes in yeast requires both transformation and subsequent maintenance of the DNA of interest. In order to efficiently maintain plasmids, without integrating them, it is beneficial to score for their presence via a selectable marker. In contrast to the plethora of markers available for *S.cerevisiae*, to our knowledge, there are currently only three commonly used marker loci in *S.pombe*, *ade6*, *leu1* and *ura4*. Of these, only those complementing the auxotrophic mutants *leu1-32 and ura4-D18*, *S.cerevisiae LEU2* and *URA3* or *S.pombe ura4+* respectively, are regularly used on multicopy vectors.

A system for gene deletion/disruption utilising *S.pombe ura4+* has been previously described (6) and a series of vectors and genomic libraries carrying *ura4+* have been constructed (7–9). Clearly, use of *ura4+* for one purpose abolishes its use for another in the same strain. Subsequently, it is easy to envisage instances where selection loci become exhausted.

Here we report the construction of a new marker system for *S.pombe* based on the *arg3+* gene (10) and employment of *ura4+* as a removable selective marker for integration/deletion.

A 1783 bp fragment of *arg3+* (–140 to +1642) was synthesised from pCVH3 (10) template using 10 cycle polymerase chain reaction (PCR), then ligated into pNEB193 (New England Biolabs), a pUC19 derivative, containing an *S.pombe ars1+* origin, Figure 1A. Six independent clones were isolated and transformed into the strain *arg3-15* to select for a functional *arg3* reading frame. Media and methods were according to (5). L-arginine (1 mg/ml) was added where required. Five of six conferred arginine prototrophy on *arg3-15*. A functional derivative was selected and called paR3.

To exclude gene conversion events at the *arg3-15* locus and thus create an ideal strain for paR3 it was decided to delete the corresponding *arg3* region from the genome. The *Hin*dIII fragment of *ura4+* is frequently used to select for gene disruption events. The *ura4+* 'pop-out' cassette shown in Figure 1B was devised for the purpose of selecting integrative transformants but allowing its subsequent removal and thus retaining the use of *ura4* as a selectable marker for ensuing experiments in the same strain. By flanking the *ura4+* fragment with 350 bp tandem repeats of pBR322 (11,12) we hoped to encourage 'pop-out' of the *ura4+* marker via intramolecular recombination between the pBR322 sequences.

To delete the *arg3* marker region, sequences flanking it were constructed by 10 cycle PCR from pCVH3 and the *ura4+* 'pop-out' cassette was inserted between them, to create pNEBarg3Δ 'pop', Figure 1B. After linearisation, the fragment was transformed into the strain *ura4-D18* and transformants were selected for uracil prototrophy. Fifty colonies were picked and restreaked on selective media. After replica plating to arginine-deficient media, one arginine auxotroph was detected. The low frequency of legitimate integration probably reflects the limited *arg3+* flanking sequence homology. To stimulate excision of the *ura4+* gene, the isolate was grown overnight in non-selective YES, washed, and ~5 × 10^5 cells were plated on 5-FOA containing media (6) to select for loss of *ura4* activity. Hundreds of colonies were obtained. To exclude isolation of spontaneous mutants in the *ura4* or *ura5* genes and to confirm 'pop-out' 20 colonies were subjected to 30 cycle PCR across the *arg3+* locus. Nineteen gave a band of ~780 bp, the expected size of a 'pop-out' event (data not shown). Chromosomal DNA was then prepared from an *arg3* wild-type strain and one of the 'pop-out' isolates. The *arg3* locus [and *nmt1* (13) as a PCR control] was again amplified by PCR and electrophoresed uncut or cut with *Eco*RV (*Eco*RI for *nmt1*) to confirm amplification of the correct locus, Figure 2A shows the predicted result of amplification and restriction digestion. The result is displayed in Figure 2B. As expected the wild-type *arg3* amplicon is 2125 bp as opposed to the deleted *arg3* of 690 bp (flanking sequences + pBR322 350 bp scar). Further, deleting the marker region removes all the *Eco*RV sites within the amplified region of wild-type *arg3* and therefore cutting *Eco*RV results in the generation of two bands contrasting to the four bands of the wild-type. Two bands are obtained with the deleted PCR band due to the presence of a single *Eco*RV site in the pBR sequence remaining in the genome. Having confirmed the DNA status of the *arg3* locus this new strain was named *arg3-D4*.

arg3-D4 was efficiently transformed with paR3, thus paR3 can complement the *arg3-D4* null allele, validating the selection system. paR3 can be easily adapted as an expression vector by insertion of a promoter and gene of choice into a combination of

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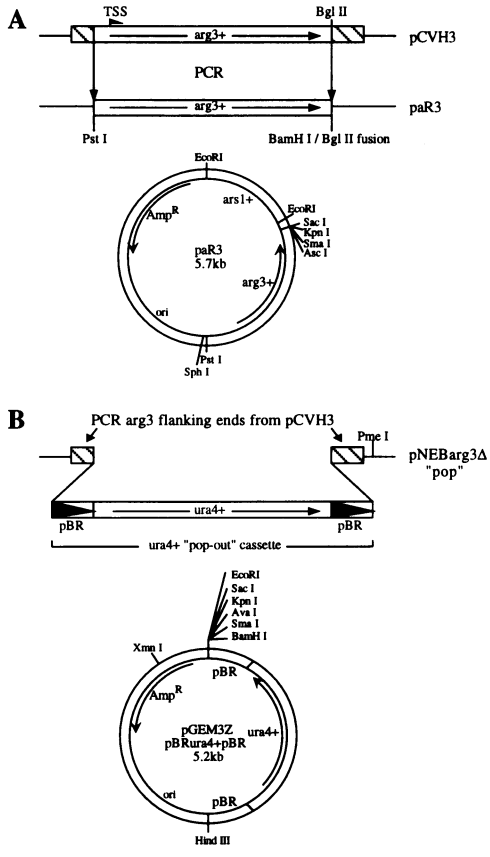

Figure 1. (**A**) Schematic representation of construction and map of paR3. A 1783 bp fragment of *arg3* from pCVH3 was amplified by PCR with *Pst*I and *Bgl*II ends and ligated into pNEB193 *ars1*+ with *Pst*I and *Bam*HI ends to give paR3. Unique restriction sites suitable for promoter/gene insertion are indicated. TSS indicates the transcript start site. (**B**) pNEBarg3Δ 'pop' construct produced for *arg3* deletion. *arg3* flanking sequences (180 bp 5′ and 320 bp 3′) were PCR'd with internal *Eco*RV and *Bgl*II sites and cloned into pNEB193. A *Hin*dIII blunt *Bam*HI fragment from pGEM3ZpBRura4+pBR was then ligated in between the *arg3* flanking sequences giving the deletion construct pNEBarg3Δ 'pop'. The pNEB193 plasmid retains a unique *Pme*I site for linearisation prior to integrative transformation. The total size of the ura4+ 'pop-out' cassette is 2500 bp. Unique restriction enzymes which excise the cassette are shown. Inclusion of *Xmn*I in restriction digestion, which cuts within the ampicillin resistance gene, aids purification of the 'pop-out' cassette from the remainder of pGEM3Z.

unique restriction sites that remain from the progenitor pNEB193. Promoter bearing variants of paR3 have been constructed (data not shown). Coordinated use of paR3 and an *arg3-D4* background will negate gene conversion at the *arg3* locus and thus favour retention of paR3 as an episome due to the complete lack of sequence homology between vector and genome. The *arg3* fragment in paR3 may be of use as an integration marker however, the ability to complement *arg3-D4* as a single copy has yet to be determined.

The ura4+ 'pop-out' cassette has also been used to integrate foreign genes at both the *arg3* and *leu1* loci (14). Selection for both integration and removal of the cassette is simply performed. It should be noted that a 350 bp pBR322 scar of the cassette is left in the genome following excision of the *ura4+* marker. This is unlikely to cause complications as a single copy however, littering the genome with multiple scars may be deleterious via recombinational exchange.

In conclusion, this report describes the construction of a new marker system for *S.pombe* based on the *arg3* gene. Additionally,

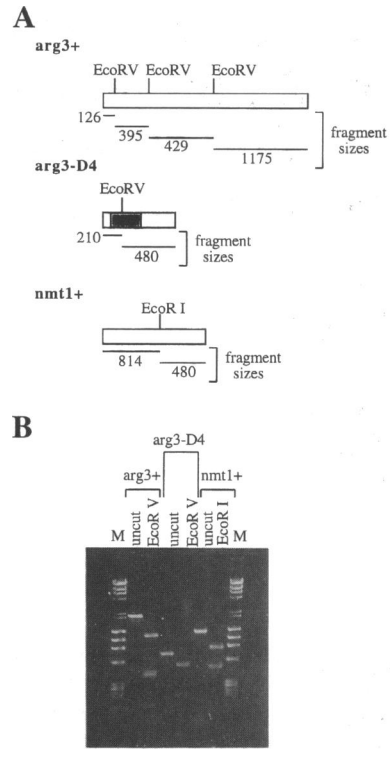

Figure 2. Confirmation of the *arg3-D4* locus genomic structure. (**A**) Predicted amplicons and restriction fragments from *arg3+*, *arg3-D4* and *nmt1+*. Sizes are given in nucleotides. The hatched area in the *arg3-D4* amplicon represents the pBR322 scar. (**B**) 1% agarose gel electrophoresis of PCR fragments uncut or cut with the restriction enzymes indicated. M-DNA standards; λ *Hin*dIII and ϕX174 *Hae*III. All fragment patterns obtained approximate to those predicted in (A). The 395 and 429 bp *Eco*RV fragments from *arg3-D4* appear as a doublet. The 126 bp fragment is visible but abolished following figure reproduction.

we have demonstrated a novel adaptation to the use of *ura4+* as a marker for integration events that permits subsequent removal of the marker and thus retains *ura4* use for future manipulations in the same strain. We believe these systems, available upon request, enhance the possibilities of molecular genetic manipulation in fission yeast and will provide a significant addition to the marker systems currently used in many laboratories.

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