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A simple and effective chromosome modification method for large-scale deletion of genome sequences and identification of essential genes in fission yeast

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ABSTRACT

The technologies for chromosome modification developed to date are not satisfactorily universal, owing to the typical requirements for special enzymes and sequences. In the present report, we propose a new approach for chromosome modification in *Schizosaccharomyces pombe* that does not involve any special enzymes or sequences. This method, designated the 'Latour system', has wide applicability with extremely high efficiency, although both the basic principle and the operation are very simple. We demonstrate the ability of the Latour system to discriminate essential genes, with a long chromosomal area of 100 kb containing 33 genes deleted simultaneously and efficiently. Since no foreign sequences are retained after deletion using the Latour system, this system can be repeatedly applied at other sites. Provided that a negative selectable marker is available, the Latour system relies solely upon homologous recombination, which is highly conserved in living organisms. For this reason, it is expected that the system will be applicable to various yeasts.

INTRODUCTION

Chromosomal engineering is emerging as an increasingly important field in the post-genomic era. This technology is of extreme importance to various fields, such as molecular biology, basic medicine and agricultural engineering, that require the development of living organisms with chromosomes carrying desired modifications for gene disruption, foreign gene insertion and mutagenesis. Various technologies for chromosomal modification have been developed to date, including the λ-red recombination system (1), the Cre/loxP system (2), the Flp/FRT system (3) and a gene replacement method involving meganuclease (4). Each of these methods has its own unique set of characteristics. However, aspects of these methods are also unsatisfactory, such as the potential for insertion of foreign sequences that remain after chromosome modification and the difficulty associated with determining the optimal conditions for enzyme expression. Moreover, all of these methods require specific enzymes and sequences as well as a substantial amount of time, and two or more steps are generally required to obtain the modified target strain.

In the present study, we developed a novel method for chromosomal modification in *Schizosaccharomyces pombe*. The resulting system, designated the latency to universal rescue system or 'Latour system', is an extremely simple method, that only requires a negative selectable marker for its application. No foreign sequences remain following chromosomal modification, and the method can be widely applied with an efficiency equal to or exceeding that of previous methods. As a concrete example of these characteristics, we not only present results that demonstrate chromosomal modification, but are also able to confirm whether or not a gene is essential for growth and very easily deleted a long chromosome area of 100 kb.

MATERIALS AND METHODS

S.pombe strains, transformation and growth conditions

All the *S.pombe* strains used in the present study were derived from MGF300 (Table 1, parental strain). Cultures were grown in YES medium [yeast extract with supplements, containing 0.5% Bacto yeast extract, 3% glucose and SP supplements (Qbiogene)]. DNA was transfected into *S.pombe* cells using lithium acetate methods, as described previously (5). The cells were plated on minimal medium (MMA; Qbiogene). When necessary, 0.01% (w/v) of supplements (uracil and/or leucine) and/or 0.05% (w/v) of 5-fluoroorotic acid (5-FOA) were added

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

Strain	Category	Genotype	Source
MGF300	Parental strain	*h-, ura4-D18*	Laboratory stock
MGF375	Latent strain	*h-, ura4-D18, Δupstream of leu1::ura4+*	This study
MGF376	Deletant	*h-, ura4-D18, leu1-D13*	This study
MGF377	Mutant	*h-, ura4-D18, Δupstream of leu1::ura4-*	This study
MGF387	Latent strain	*h-, ura4-D18, Δupstream of ch.1-100K::ura4+*	This study
MGF388	Deletant	*h-, ura4-D18, ch.1-100K-D1000*	This study

to the MMA. Growth of *S.pombe* strains was performed at 30°C.

PCR

DNA fragments were amplified by PCR using a GeneAmp PCR System 9700 (Applied Biosystems). Each amplification was completed with *ExTaq* (TaKaRa) or KOD Dash (Toyobo) DNA polymerase. The general conditions used were an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 30 s to 3 min, depending on the length of the DNA to be amplified. The PCR products were gel-purified and recovered using a gel purification kit (Qiagen). The quality and concentration of the DNA products were analyzed by gel electrophoresis and UV spectrophotometry, respectively.

Generation of targeting fragments

Two DNA targeting fragments were generated by fusion PCR (6). The flanking regions (400 bp) and direct repeats (200 bp) were amplified using primers specific for the upstream and downstream regions of the target genes. The flanking regions for *leu1* are nt 358 934–359 353 and nt 359 334–359 733 of the second chromosome. The direct repeats sequence for *leu1* is nt 357 618–357 817 of the second chromosome. The flanking regions for 100 kb are nt 5 412 809–5 413 208 and nt 5 415 172–5 415 571 of the first chromosome. The direct repeats sequence for 100 kb is nt 5 513 211–5 513 410 of the first chromosome. The entire list of primers used for the construction of the targeting fragments can be found as Table 2.

Construction of latent strains for *leu1* and 100 kb

Latent strains for *leu1* and 100 kb were constructed by transfection of targeting fragments (generated by fusion PCR as described above). Clones with the appropriate homologous recombination event were selected by PCR (check primers on Table 2) and sequencing of their genomic DNA. The genomic DNAs were purified using a DNeasy Tissue Kit (Qiagen).

Deletant and mutant strains derived from the latent strains by 5-FOA treatment

Latent strains from the parental strain were carefully isolated on medium without uracil. Since the uracil auxotrophy of the parental strain was the same as those of the deletant and mutant strains, this process was repeated at least three times. The isolated latent strains were cultured in YES medium for 2–3 days at 30°C, and then plated at $\sim 10^6$–10^8 cells on MMA medium containing 5-FOA. If the target gene/region was non-essential, deletants appeared by homologous recombination between direct repeats during the cultivation in YES medium, an $\sim 10^{-5}$–10^{-7} chance.

Table 2. Primers for generation of targeting fragments

Primers		Sequence (5′–3′)
leu1 upstream	Fw	aaagaggccaaccagaagag
leu1 upstream	Rv	ttattctacattaaaccctaaaatttttaatgtcaaaaaa
leu1 downstream	Fw	tttcgtcaatatcacaagctcgtttactaacgtagaaagc
leu1 downstream	Rv	gttgttgaagaagttttgtt
leu1 check	Fw	aagatgacgatgatgatttt
leu1 check	Rv	gtcgcttcttctcaacgact
leu1 direct repeat	Fw	tagggtttaatgtagaataa
leu1 direct repeat	Rv	gtgggatttgtagctaagctggatgtcgtaaatcaattcc
leu1 check	Rv	agagagcagcccgcgatagc
100K upstream	Fw	agaattgagacggcgctgaa
100K upstream	Rv	gtccttttgttaaataaaaattaggatacactaggtagat
100K downstream	Fw	tttcgtcaatatcacaagcttgttgctttttatattaaa
100K downstream	Rv	aaacaagactaaagattagt
100K check	Fw	gacagtaaaagcattaagta
100K check	Rv	gctttaccaacttcgtcaga
100K direct repeat	Fw	tttttatttaacaaaaggac
100K direct repeat	Rv	gtgggatttgtagctaagcttttatcgaaagaaaagaaat
100K check	Rv	tcttagataattgacagaac

RESULTS

New chromosomal modification method using recycling of a selectable marker

One of the most common methods for transformant selection is using uracil complementation in yeast as an index, and the orotidine 5′-phosphate decarboxylase gene as a selectable marker. This selectable marker can be utilized for both positive and negative selection (7). Uracil autotrophs are selected in a uracil-deficient medium, while uracil auxotrophs are selected in a medium containing 5-FOA. The 5-FOA is an analog of a uracil precursor and fluorouracil synthesized from 5-FOA has the potential to strongly inhibit yeast growth. Therefore, uracil autotrophs cannot grow in the presence of 5-FOA.

Since this selectable marker (*URA3*) is recycled in *Saccharomyces cerevisiae* and *Candida albicans*, there is a method for deleting *URA3* from the chromosome (8,9). In this method, *URA3* is deleted by homologous recombination, following the introduction of a integration fragment with direct repeats arranged previously both upstream and downstream of *URA3* (Figure 1A).

However, upon reflection, the direct repeats are only required for the marker rescue ‘after’ the modification fragment to be introduced is integrated into the chromosome by double crossover. Therefore, given a modification fragment such as that shown in Figure 1B, even without inclusion of direct repeats in the modification fragment, direct repeats are located both upstream and downstream of the selectable marker on the chromosome following integration. If a region further downstream from the target sequence is then designed to be the direct repeats, we hypothesized that it would be

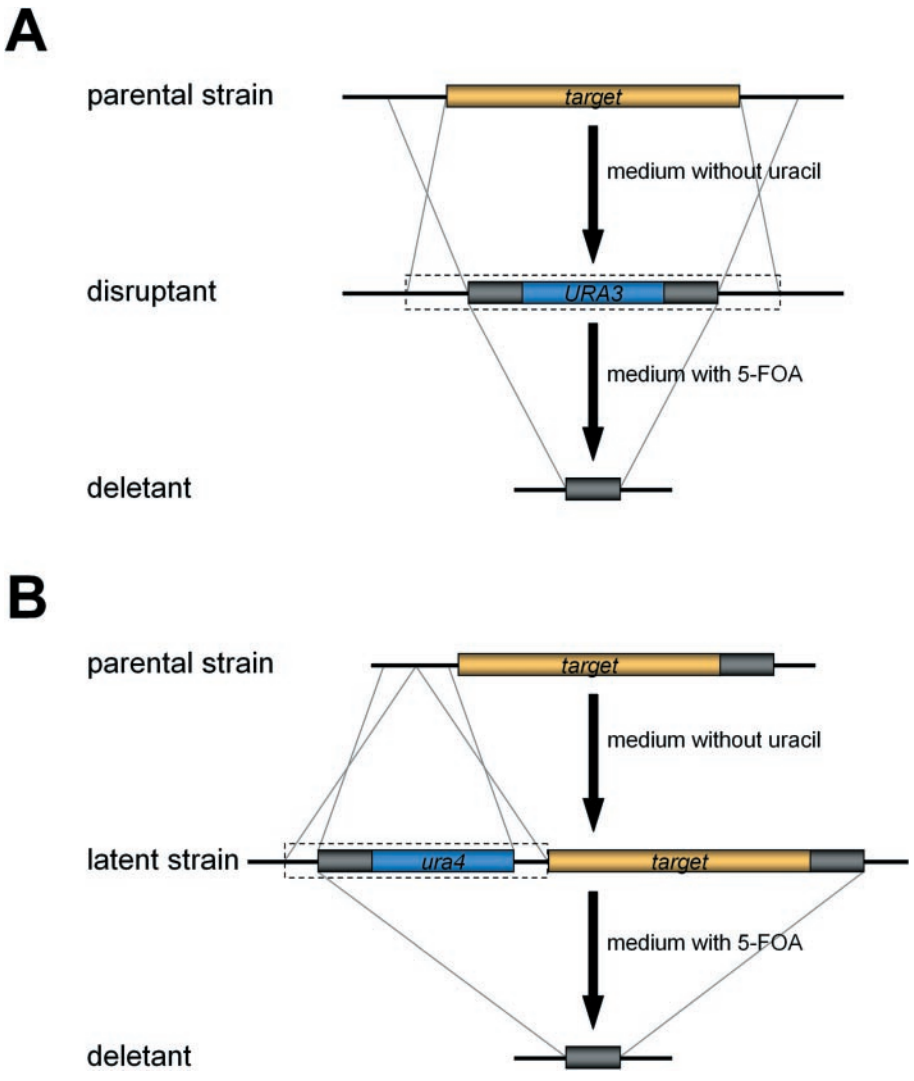

Figure 1. Schematic representation of the deletion (recycling) of the selectable marker using direct repeats. The gray squares show the direct repeats. The portions enclosed with a dotted line show the introduced modification fragments. (**A**) *URA3* recycling methods that have been developed previously in *S.cerevisiae* and *C.albicans*. Note that the direct repeats are in the inserted modification fragment. (**B**) The chromosomal modification method developed in this study. The direct repeats are not contained in the modification fragment, and the *ura4* and target genes are placed between direct repeats on the chromosome during the latent stage. The important difference from previous methods is that the target gene to be deleted is retained during the stage in which the modification fragment is introduced.

possible to delete both the target sequence and the selectable marker by 5-FOA treatment (Figure 1B).

Production of a gene-deleted strain with *leu1* as the target gene

We confirmed the feasibility of deleting a target sequence and selectable marker by this method in *S.pombe*, using the 3-isopropylmalate dehydrogenase gene (*leu1*) as the target gene (Figure 2). A strain without *leu1* can grow in a medium containing leucine, but cannot grow in a completely leucine-deficient medium. We therefore consider that the *leu1* gene is an essential gene for growth in a medium without leucine, but a non-essential gene for growth in a medium containing leucine.

First, we produced a strain in which the region upstream of *leu1* was modified (latent strain) by the presence of an orotidine 5′-phosphate decarboxylase gene (*ura4*). Both *ura4* and a sequence 200 bp downstream from *leu1* were located in the introduced modification fragment. Although the fragment to be introduced did not itself contain direct repeats, *ura4* and the target gene *leu1* were located between direct repeats following integration of the modification fragment into the chromosome (Figure 2A).

By growing this modified latent strain in a medium containing 5-FOA, 5-FOA-resistant colonies were formed. In eight of these 5-FOA-resistant strains, PCR was performed to determine whether or not they represented target gene-deleted strains. As a result, bands shorter than those characteristic of the latent strain were obtained for all eight strains using primers for both flanking regions (Figure 2B, left panel). In addition, amplification of the open reading frames (ORFs) of *ura4* and *leu1* was not observed using primers specific for amplification of the ORFs (Figure 2B, left panel). In confirmation of the auxotrophy, all eight strains were both uracil and leucine auxotrophy (data not shown). Moreover, sequence

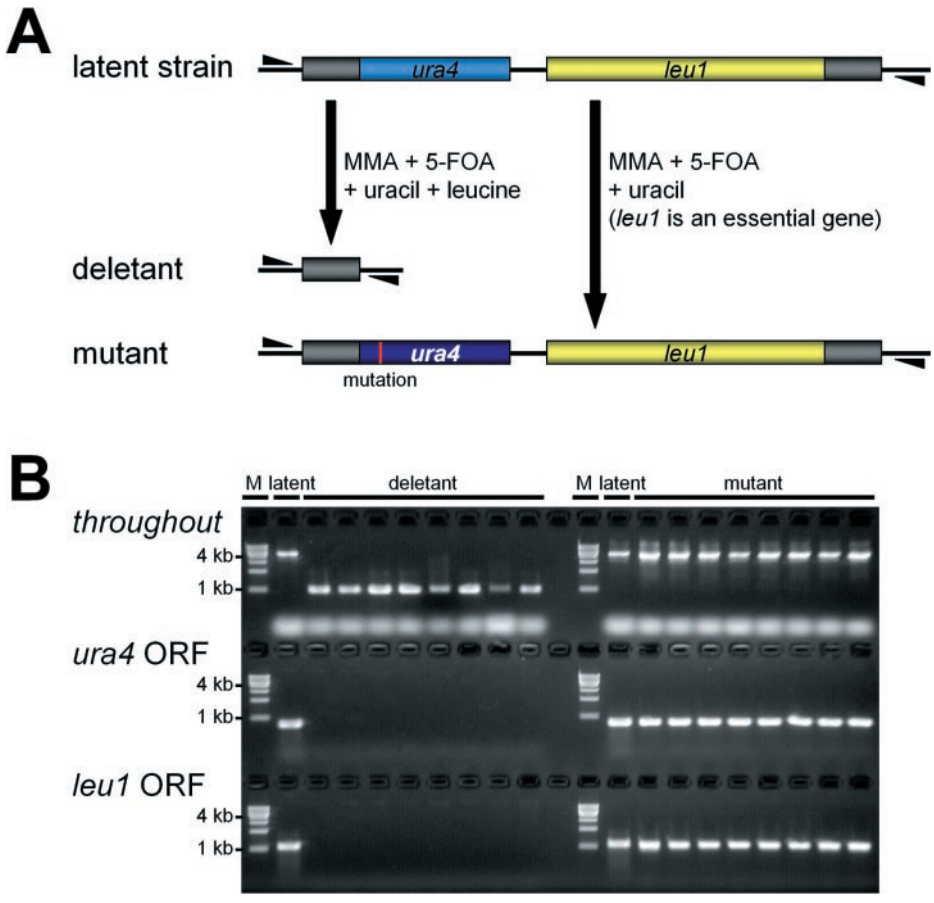

Figure 2. Examples establishing the Latour system involving the *leu1* gene. (**A**) Schematic representation of chromosomal modifications that can occur by 5-FOA treatment of a latent strain. Gray squares show the direct repeats, while closed triangles show the primers used for 'throughout' in Figure 2B. In the medium containing leucine, *leu1* and *ura4* are deleted by homologous recombination because the *leu1* gene is not required, such that a deletant strain is obtained. On the other hand, in the situation in which *leu1* is essential (i.e. in the medium without leucine), mutations occur in *ura4* with retention of *leu1*, such that a mutant strain without *ura4* activity is obtained. (**B**) Identification of sequences by PCR. Left panel, situation in which *leu1* is not required (i.e. medium containing leucine). Right panel, situation in which leu1 is essential (i.e. medium without leucine). When the *ura4* and *leu1* sequences have been deleted, a region of ~1000 bp is amplified in 'throughout', but neither of the ORFs (*ura4*, 795 bp; *leu1*, 1116 bp) are amplified (left panel). On the other hand, when both sequences are retained, a sequence of ~4000 bp is amplified in 'throughout', and the ORFs of both *ura4* and *leu1* are also amplified (right panel). M is a DNA molecular size marker (1 kb DNA Ladder, Toyobo).

analyses clarified that the regions upstream and downstream of the target sequence had been seamlessly integrated without leaving even a single base of foreign sequence (data not shown). These results confirm the generation of strains in which both *ura4* and the target gene *leu1* were deleted (deletant, Figure 2A) with high efficiency in 5-FOA medium, by selecting the latent strain integrating *ura4* and the direct repeats upstream of *leu1*.

Requirement of the target sequence for growth results in *ura4* mutations

In the example described above, the *leu1* gene was not required for growth, since the medium contained leucine. It was also important to investigate the situation in which the *leu1* gene is essential for growth (i.e. in a medium without leucine). Strains that are able to synthesize uracil (i.e. uracil autotrophs) cannot grow in 5-FOA medium. On the other hand, if the *ura4* and *leu1* genes are deleted, the resulting strains cannot grow, since leucine is not synthesized. Therefore, in a 5-FOA medium without leucine, it is expected that only mutant strains that lack *ura4* activity due to mutation but retain *leu1* will appear (Figure 2A).

Results confirming this expectation are illustrated on the right side of Figure 2B. By plating the latent strain on a 5-FOA medium without leucine, 5-FOA-resistant colonies were formed. PCR analysis of eight strains from these colonies detected bands with similar mobilities to those of the latent strain using primers designed to amplify the sequences flanking the direct repeats and the ORFs of *ura4* and *leu1*. These results indicate that the sequence obtained was that shown in Figure 2A. Regarding auxotrophy, the latent strains were both uracil and leucine autotroph. However, all eight mutant strains were leucine autotrophy but uracil auxotrophy (data not shown). Moreover, sequence analyses revealed amino acid substitution mutations in the *ura4* ORFs of all eight strains (D227Y, D227Y, D227N, T121K, S33R, T121R, T121K and T121R).

In the situation in which *leu1* was not required (i.e. in a medium containing leucine), both the *ura4* and *leu1* genes were deleted by homologous recombination with extremely high efficiency. On the other hand, in the situation in which

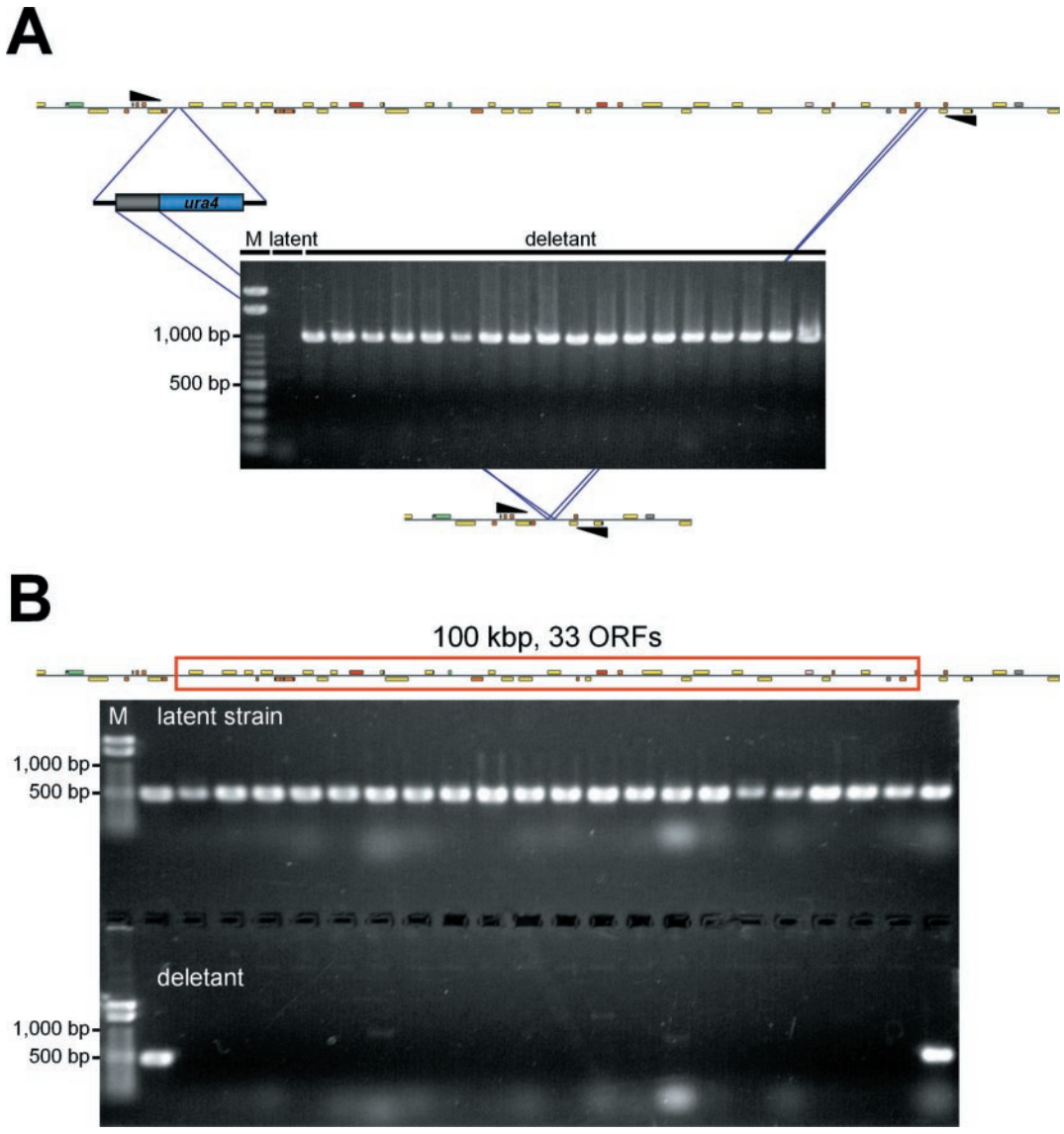

Figure 3. Schematic representation of the characteristics of the Latour system, and an example application of the system, in which a region of 100 kb is deleted. (**A**) Schematic representation of the gene structure near the right telomere arm of the first chromosome, and PCR results of the 100 kb deletion. Closed triangles show the primers used to confirm the deletion. In the latent strain, amplification by PCR is not observed due to the very long length of the 100 kb band. On the other hand, in the 5-FOA-resistant strains, 1000 bp bands are amplified, indicating that the 100 kb sequence has been deleted in all 18 strains. M is a DNA molecular size marker (100 bp DNA Ladder, Toyobo). (**B**) Confirmation of the ORFs by PCR. The presence/absence of the ORFs is confirmed by primer amplification of a 500 bp sequence in 20 of the 33 ORFs included in the 100 kb target sequence of the first chromosome, and in ORFs in the regions adjacent to both ends of the target sequence. The band is amplified in all the latent strains, but not in the deletant treated with 5-FOA, except for ORFs in the adjacent areas of the target area, indicating that the 100 kb target has been deleted. The entire list of primers for this confirmation of the ORFs can be found as Table 3. M is a DNA molecular size marker (100 bp DNA Ladder; Toyobo).

leu1 was essential for growth (i.e. in a medium without leucine), it was determined that the activity of *ura4* had disappeared due to point mutations, and that the *leu1* gene had been retained. We are convinced that it is useful to assess whether a gene or area of the chromosome is essential for growth, if this technique is to be applied (for details see Discussion).

Deletion of a long chromosomal area of 100 kb is possible with extremely high efficiency

The characteristics of the Latour system are such that the target sequence is retained (Figure 1B) during the stage when *ura4* and the direct repeats are latent on the chromosome, and that *ura4* and the target sequence can be deleted with extremely high efficiency if they are in an area that can be deleted. Moreover, another important characteristic is that the insertion position of the modification fragment can be selected as desired, since the direct repeats are separated on the modification fragment and the chromosome. Using this characteristic, the latent strain can be produced by the previously established double crossover method of single gene disruption, regardless of the length of the target area (Figure 3A).

As an example of efficient usage of the Latour system, we present the results for successful deletion of a

Table 3. Primers for this confirmation of the ORFs

Gene name	Forward primer	Reverse primer
ura4	atggatgctagagtatttca	ttaatgctgagaaagtcttt
leu1	atgtgtgcaaagaaaatcgt	ttacaaaatttttcaagtt
SPAC29B12.14	atgccaaaatggagattgac	gagggccaaatggatcgaat
SPAC1039.01	atgtcgtctattatggaaaa	ttaacagatggctgtccaat
SPAC1039.02	atgaagttatcatctttacc	taaatttgaacgttagaagc
SPAC1039.04	atggagaaatctatatcatc	aaaactgctgactctgccat
SPAC1039.05	atgaccaaggcgaaaaaaag	gaaattatcggaaattatgg
SPAC1039.07	atgagttctcatgtaagtaa	gctggaccatagccgcgtcg
SPAC1039.08	atgactgtgcagacagcgga	gcgtggtcaagaaacaagcc
SPAC922.03	atgggtttagaacagtttaa	tagggatgttccgagcaacc
SPAC922.04	atgaagtttttctgggtctc	aggtgcaaatttattataat
SPAC922.06	atgactgttgaaggacgagt	cctaaaggcccatatcgaac
SPAC922.07	atgtcagaagatttatttgt	ggataattccatgggactat
SPAC869.09	atgcctgatcctgcacatat	caaggtaacgagcataacta
pcm2	atgttctggagttttaacct	gaggcacccacatgaatggc
SPAC869.06	atgcttcagtattccaagaa	acaaacttctttgttctttc
SPAC869.05	atgcgcgcttggggttgggt	aaaaagcaatatatagcaac
SPAC869.03	atgacagaaacagttcttaa	gcaacaacatgcattccagt
SPAC869.02	atgtccagtgttgaggttaa	tcagagtcaatcaaaatttt
SPAC186.01	atgaatgtggtgaagtacat	tcacctgactgtgttccaga
SPAC186.02	atgcgtgttgtccttttag	aaacccatttttaaaacattt
SPAC186.05	atgattaatgttgaaagtcc	aaacagctcatactaaagac
SPAC186.06	atgtcaatcaaacttggata	tcgtataatccgtttgcaga
SPAC186.08	atgaccgctggttctaaagt	ctctaaggatttccttaaag

long area of 100 kb to obtain a deletant, a process that is very difficult by previous methods. In the area within 100 kb of the right arm telomere of the first chromosome (nt 5 413 211–5 513 210), there are 33 ORFs and 2 non-coding RNAs. No essential genes are known to exist in this area. Furthermore, there are no genes in this area that could be presumed to be essential by homology to *S.cerevisiae*. Therefore, we produced a latent strain that integrated the *ura4* gene and direct repeats from the ORF downstream of SPAC186.06 to 100 kb upstream (Figure 3A). After selecting this latent strain using the above-described method in 5-FOA medium, strains in which 100 kb had been deleted were obtained with extremely high efficiency, as expected (Figure 3A). Sequence analysis of PCR products amplified from these deleted strains confirmed that the upstream and downstream regions of the target sequence had been integrated seamlessly, without leaving even one base of foreign sequence (data not shown). Moreover, during confirmation of the 20 ORFs of the target sequence by PCR, amplification of the ORFs was not observed in the deleted strains, and it was thus determined that the 100 kb sequence had been deleted as expected (Figure 3B). These results confirm the absence of essential genes and synthetic lethality in this area. Furthermore, a remarkable difference was not seen compared with a wild type on the growth and morphology of the 100 kb-deleted strain (data not shown). Since homologous recombination occurs efficiently even over a region as long as 100 kb, the Latour system can be efficiently applied not only to a single gene, but also to a lengthy region of 100 kb, which represents roughly 1.75% of the first chromosome.

DISCUSSION

The experimental operation of the Latour system is substantially similar to the previous gene disruption method involving double crossover, and additional operations are not specially required. Since the direct repeats originally exist on the chromosome, no foreign sequence is retained following deletion of the marker and the target gene (Figures 1B, 2A and 3A). Since foreign sequences are retained with the Cre/loxP and Flp/FRT systems, repeated use of such systems carries a greater potential for unexpected recombination. On the other hand, since no foreign sequence is retained in the Latour system after the deletion, this system can be repeatedly applied at other sites. Moreover, in the Latour system, the introduction and expression of enzymes (recombinase, meganuclease and the like) are not required for the marker rescue, in contrast to previous systems. The marker is simply deleted by negative selection of the latent strain. Although the loxP sequence is separately integrated at two sites in mice for deletion of a very long region (10), the modification of only one side of the target gene is required for deletion, without modification of the other side, in the Latour system (Figure 3A). We are therefore convinced that the Latour system represents substantial improvements in ease and effectiveness compared with previously established methods.

The other important characteristics of the Latour system are that the target gene is retained during the stage when the selectable marker is integrated (Figures 1B, 2A and 3A, latent strain), and that the deletion efficiency is extremely high (Figures 2B and 3A, deletant). Using this system, we were able to test whether or not a gene or chromosome region was essential for growth (Figure 2). We consider the *leu1* gene used in this study is an essential gene in a medium without leucine, but a non-essential gene in a medium containing leucine. Thus, in the situation in which the marker cannot be deleted by the Latour system (i.e. in which a deletant cannot be obtained), such that entry of a mutant into the marker is obtained with retention of the target gene, we can confirm that the target gene and area are essential for growth.

Modification of a gene sequence is one of the most common methods used for analyzing gene function. However, it is extremely difficult to obtain modified strains in many organisms (11), including *S.pombe*. In particular, when direct modification of a target gene with unknown function is unsuccessful, it cannot be clearly determined whether the lack of success is due to low efficiency or because the gene is essential for growth. In the Latour system, the target gene is not modified during the latent stage. As a result, it is possible to produce a latent strain regardless of whether or not the target gene is essential for growth. Through negative selection of the obtained latent strain, the strain always becomes either a deletant or a mutant (Figure 2A). This represents an easy method for clearly determining whether a deletant of the target gene is obtained or the region is essential for growth.

To check for essential genes, the widely accepted method is to delete the gene in a diploid strain and follow the haploid segregants by tetrad analysis. The Latour system also can ascertain an essential gene, but by a quite different approach from that in tetrad analysis. The manipulation is as simple and the time spent the same as in tetrad analysis. However, tetrad analysis cannot be used for *C.albicans*, which has been classified as a diploid, asexual organism (9). Since it is independent of such a characteristic, the Latour system will be applicable to *C.albicans* which recyclable marker system has already established (9).

In the situation in which a target gene is not required for growth, it is possible to grow not only a deletant, in which the

ura4 and target genes have been deleted, but also a mutant, which has entered into *ura4* with retention of the target gene. However, in our results to date, such a mutant has not been obtained (Figure 2B, left panel, and Figure 3A). Since mutations are usually undesirable for living organisms, many repair mechanisms exist for correcting mutations, such as nucleotide excision repair, base excision repair and mismatch repair (12). On the other hand, general homologous recombination is ordinarily (rather positively) performed in chromosomal double-strand break repair and meiosis (13). Homologous recombination occurs with a much higher frequency than mutations. It may therefore be considered that only the deletant is obtained in situations in which the target gene is not essential.

Operation of the Latour system is extremely simple, since it uses exactly the same procedures as the existing method for single gene disruption. The basic principle relies exclusively upon the very basic biological function of homologous recombination. Therefore, the Latour system can certainly be applied to various yeasts that can utilize 5-FOA, such as *S.cerevisiae* (8), *C.albicans* (9), *Pichia pastoris* (14) and *Kluyveromyces lactis* (15).

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