Transient Marker System for Iterative Gene Targeting of a Prototrophic Fungus

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Auxotrophic microorganisms are often used for genetic engineering, because their biosynthetic deficiency can be complemented by the transforming DNA and allows selection for transformants that have become prototrophic. However, when complementation is obtained by ectopic expression this may lead to unpredictable side effects on the phenotype and, consequently, misinterpretation of experimental data. There are various ways to overcome the problem of auxotrophy, but the most reliable is to restore the function of the defective biosynthetic gene at the native genomic locus. This can be done by either sexual crossing or further genetic engineering. For fungal species lacking a perfect state or situations in which gene targeting is generally cumbersome we have developed a concept that allows transient disruption of the gene. When the gene is in the disrupted state, multiple rounds of gene targeting can be performed with the strain. Once the desired genome engineering is completed, the prototroph function can be rapidly returned to wild type by a simple selection scheme.

Gene targeting in microorganisms is often performed with auxotrophic strains by use of a nutritional transformation marker for selection. However, once the genetic manipulation has been completed, the auxotrophy is undesired, as it may influence analysis of the engineered strain (1, 9, 20). For example, it has been shown that the pathogenicity of the plant pathogen Fusarium oxysporum (14) and that of the human pathogen Aspergillus fumigatus (4) are reduced by mutations in the arginine and a pyrimidine biosynthesis gene, respectively. In the effort to avoid working with auxotrophic strains, the marker is often left at the manipulated locus (20). However, even though this arrangement results in a prototroph, the expression level of the marker at the ectopic location may differ from that of the wild type; thus, the marker contributes to the overall phenotype and complicates further analysis (8, 10). For example, when a ura3 mutation in Candida albicans was complemented by integrating a Saccharomyces cerevisiae URA3 gene at different loci, strains displaying various levels of virulence were obtained as the result of differing gene expression levels (12). Also, for industrial strains it has been observed that random integration of genes results in greatly varying phenotypes such as different production levels of the desired product (27). Moreover, it is easy to imagine that conclusions drawn from data obtained by high-throughput methods will be particularly vulnerable to this type of experimental artifact(s).

Another way to solve the auxotrophy problem is to add the required nutrient to the medium. However, titration of the lacking nutrient may be necessary to determine which concentration is required to obtain the same phenotype as the prototroph, and this assessment is difficult for uncharacterized mutant strains (7). Moreover, in the case of industrial fermentations, auxotrophies are undesired since addition of supplements to the media increases production cost. In light of these disadvantages, it is generally advisable to eliminate the selectable marker at the engineered locus and restore the auxotrophic marker to wild-type status before further experimentation (reviewed in reference 20). This can be accomplished by creating the genetic modifications by use of a two-step gene-targeting method where the selectable marker is eliminated in the second step after the actual gene targeting has been done (22). At this stage, the auxotrophic marker can be reintroduced, either by time consuming sexual back-crossing or alternatively by restoring the defective gene by additional gene targeting. However, in species without a sexual cycle or situations in which gene targeting is tedious, these options are impractical. Hence, for a diverse group of organisms, like the filamentous fungi, which contains many imperfect species and for which gene targeting is generally cumbersome, a method that facilitates the restoration of the auxotrophic marker would be beneficial.

Recently, we introduced a gene-targeting system in which a DNA repair gene was transiently disrupted in order to improve targeting efficiency without compromising DNA repair fidelity in the final strain (16). Inspired by this work we addressed the problem of working with auxotrophic strains by constructing a transiently disrupted nutritional marker, which can revert to wild type when its role in genetic engineering is complete. For proof of concept, A. niger, the industrially relevant filamentous fungus, was used as a model organism. The gene pyrG, encoding orotidine 5'-phosphate decarboxylase, was chosen as the target for transient disruption, as this permitted both selection and counterselection of the complementing transformation marker and thus facilitated two-step gene-targeting procedures (17, 19). In the resulting strain, the acuB gene was successfully deleted using a recyclable gene-targeting cassette (17), and subsequently the pyrG locus was reverted to wild type.
TABLE 1. Oligonucleotides used in the study

<table>
<thead>
<tr>
<th>Code</th>
<th>Usage</th>
<th>Sequencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1</td>
<td>Disruption of pyrG</td>
<td>GGAACTGCCTTGGCAAGGTGTTGCG</td>
</tr>
<tr>
<td>T2</td>
<td>Deletion of acuB</td>
<td>GATCCCCCGGAAATGCGCATTACAAAGTCTCCTCTACCGAGG</td>
</tr>
<tr>
<td>T3</td>
<td>hp marker (Hyg resistance)</td>
<td>GGAAGCAGGTCGATCATCACTAAGAGCGCTACTAGTCG</td>
</tr>
<tr>
<td>T4</td>
<td>pyr-4 marker</td>
<td>GCTCCTTAGGCGGATGTCGTGTTG</td>
</tr>
<tr>
<td>A1</td>
<td>Recyclable pyr-4 marker</td>
<td>CATGGCAAATCTCCGGGGATGGCTGATTGAGTGAGCCAGAG</td>
</tr>
<tr>
<td>A2</td>
<td>Hyg marker</td>
<td>CTTGCATCACAAAGCGCAAC</td>
</tr>
<tr>
<td>A3</td>
<td>acuB upstream probe</td>
<td>CATGGAAATTCGCCGGATAGCAAACCGTACCGG</td>
</tr>
<tr>
<td>A4</td>
<td>acuB downstream probe</td>
<td>GCGCTTGTGGGGTGAAGTTGTTCC</td>
</tr>
<tr>
<td>H1</td>
<td>Deletion of pyrG</td>
<td>CATGGCAAATCTCCGGGGATGGCTGATTGAGTGAGCCAGAG</td>
</tr>
<tr>
<td>H2</td>
<td>Disruption of acuB</td>
<td>CTTGCATCACAAAGCGCAAC</td>
</tr>
<tr>
<td>H3</td>
<td>Recyclable pyr-4 marker</td>
<td>CATGGAAATTCGCCGGATAGCAAACCGTACCGG</td>
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<tr>
<td>H4</td>
<td>Hyg marker</td>
<td>GCGCTTGTGGGGTGAAGTTGTTCC</td>
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<td>M1</td>
<td>Recyclable pyr-4 marker</td>
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<td>P1</td>
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<tr>
<td>P2</td>
<td>acuB downstream probe</td>
<td>GCGCTTGTGGGGTGAAGTTGTTCC</td>
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</table>

a All sequences are shown in 5'-to-3' direction. Sections of the oligonucleotides in bold represent fusion tags.

MATERIALS AND METHODS

Strains, plasmids, and PCR. A. niger N402 (FGSC A733) was used as wild-type strain and was the basis for strain constructions. Plasmid pDEL1 contains pyr-4 of Neurospora crassa flanked by a direct repeat (DR) (17). Plasmid pAN7-1 harbors the bacterial hph gene, conferring hygromycin resistance, under the control of the A. nidulans gpdA promoter and trpC terminator (21). Oligonucleotides (MWG Biotech) used in this study are listed in Table 1. PCR was performed as described previously for A. niger (17). Oligonucleotides were used to fuse corresponding targeting and marker sequences to create the bipartite substrate.

Selection for the excision of the pyr-4 cassette was done by plating 2 × 106 to 3 × 108 spores on 5-FOA medium.

Southern blot hybridization. For each strain, approximately 2 μg of genomic DNA was isolated and digested with appropriate restriction enzymes (17). Sequence information for restriction digestion of the pyrG and acuB loci was obtained from the A. niger ATCC 1015 genome sequence data provided by the U.S. Department of Energy Joint Genome Institute (http://genome.jgi-psf.org/Aspni1/). Blotting was done according to the method described by Sambrook and Russell (24) by use of RapidHyb hybridization buffer (Amersham Pharmacia) for probing. The pyrG locus was detected by probing with the labeled 570 bp PCR fragment (oligonucleotides T3 and T2 on genomic DNA) of the DR of pyrG. Similarly, the 1 kb acuB upstream probe was generated by PCR (oligonucleotides P1 and P2). The probes were radioactively labeled with [α-32P]dCTP by random priming using a Rediprime II kit (GE Healthcare).

Assessing the stability of the direct repeat in the transiently disrupted pyrG strain. To estimate mitotic recombination frequencies for the DR sequences of the transient pyrG strain, nine independent trials were performed. As a standard, colonies were grown to a diameter of 2 cm and all conidiospores were harvested. Approximately 200 spores were plated on MM–Ura–Uri to evaluate the germination efficiency of the strain, and a dilution series of the remaining spores was plated on MM to select for recombinants. After incubation at 30°C for 4 days, colonies were counted and DR recombination frequencies were calculated for each strain. The recombination frequency presented is the median number calculated from the results obtained in the nine trials.

Nucleotide sequence accession number. The N402 derivative containing pyrG ∆, the transiently disrupted pyrG locus, which was constructed in this study, was deposited in GenBank (accession no. X06626).

TABLE 2. PCR fragments for bipartite gene-targeting substrate construction

<table>
<thead>
<tr>
<th>Final strain and substrate part</th>
<th>Oligonucleotides used for:</th>
<th>Targeting fragments</th>
<th>Marker fragments</th>
</tr>
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<tbody>
<tr>
<td>pyrGΔ</td>
<td>A</td>
<td>T1 + T2</td>
<td>H1 + H2</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>T3 + T4</td>
<td>H3 + H4</td>
</tr>
<tr>
<td>acuBΔ</td>
<td>A</td>
<td>A1 + A2</td>
<td>M1 + M2</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>A3 + A4</td>
<td>M3 + M4</td>
</tr>
</tbody>
</table>

a See Table 1 for oligonucleotide details. Italicized codes represent primers used to fuse corresponding targeting and marker sequences to create the bipartite substrate by PCR.
colonies were all sensitive to Hyg, showing that they had lost hph, likely due to a DR recombination event (Fig. 1B). For successful use of pyrG as a selectable marker for gene targeting, the stability of pyrG, is important, since reconstitution of pyrG during gene targeting results in false positives on the selective plates. To assess the impact of this, we determined the frequency of spontaneous prototroph formation for a randomly selected pyrG strain. We observed that prototrophs developed at a frequency of $1.5 \times 10^{-4}$. Hence, since approximately $10^6$ protoplasts are used during transformation, it is advisable to replicate transformants in medium containing Hyg to select for true transformants. Alternatively, a more stable disruption may be constructed by reducing the length of the pyrG-based DR (16).

**Successful gene deletion in the transiently pyrG-disrupted strain.** To delete the entire open reading frame (ORF) of acuB in a pyrG strain, we used a bipartite gene-targeting substrate made solely by PCR (see Materials and Methods). During a correct integration event, the bipartite gene-targeting substrate is joined by recombination, and the resulting pyr-4 cassette replaces the acuB ORF and transformants can be screened for uracil prototrophy and loss of AcuB activity.

It had previously been found that disruptants of acuB are able to grow on propionate but not on acetate as a sole carbon source (23, 25). Moreover, it was observed that propionate interferes with sporulation and growth of strains able to use acetate (25). Accordingly, since acuB-positive strains fail to sporulate on propionate medium, acuBΔ strains can be identified simply as strains that sporulate on this medium. After transformation, 250 colonies were obtained and these were vortexed and plated on propionate medium. Ten patches were found to sporulate, and spores were randomly picked for further screening. Six of these colonies showed severely retarded growth on acetate medium compared to wild-type results and yet retained the ability to grow on propionate and Hyg plates. These transformants were candidates for correctly targeted strains.

The next step was to excise the pyr-4 marker, which had replaced the acuB ORF. Since the enzyme orotidine 5'-phosphate decarboxylase encoded by pyrG and pyr-4 converts 5-FOA into a cytotoxic compound, 5-FOA-containing medium only allows growth of cells lacking this enzyme. Therefore, selection can be made for the infrequent cells that have lost pyr-4 (the native pyrG is still disrupted by hph) due to recombination between the repeated sequences flanking pyr-4 (Fig. 1B). Accordingly, around $10^6$ conidia from the acuBΔ::pyr-4 strains were plated on 5-FOA medium to ensure that a significant number of 5-FOA-resistant colonies were present, indicating that they had lost Pyr-4 activity (17). As expected, these colonies were all Hyg resistant, indicating that the transient disruption (pyrGΔ) was still in place in the native pyrG locus. If desired, additional gene targeting can be performed at this stage of the strain construction process by use of gene-targeting substrates based on the pyr-4 cassette.

**Reconstitution of native pyrG after successful genome manipulations.** Since the aim of this project was to construct a strain that solely contained an acuB deletion, approximately $10^6$ conidia from acuBΔ pyrGΔ strains were plated on MM to select for the reconstitution of pyrG due to the loss of the disrupting hph gene. The colonies that developed were tested

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**RESULTS AND DISCUSSION**

**Transient disruption of pyrG.** Engineering of pyrG was accomplished by transforming protoplasts with a bipartite gene-targeting substrate (17) and hph, the dominant selection marker that confers resistance to hygromycin B. To construct pyrG, representing a transient pyrG disruption, we used the upstream and downstream DNA fragments to target pyrG; these fragments share 570 bp of common pyrG sequence that forms a DR once the bipartite gene-targeting substrate has integrated at the genomic locus (see Fig. 1A). Since the repeated sequences flank the Hyg resistance gene, pyrG can be made to revert to the original pyrG sequence via a DR recombination event (see Fig. 1B). A total of 156 transformants were obtained, and out of 50 tested colonies, 30 were both resistant to Hyg and unable to grow without supplementation of uracil, indicating that pyrG had been successfully disrupted in these strains. Once constructed, the pyrGΔ strain can be used as the starting point for further genomic manipulations.

When large numbers of spores ($2 \times 10^6$ to $3 \times 10^6$) from each of the pyrGΔ strains were plated on MM, a few colonies always appeared after 4 days incubation, indicating that pyrG function can be restored at low frequency. Additionally, these spores were all resistant to Hyg, showing that they had lost hph, likely due to a DR recombination event (Fig. 1B).

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FIG. 2. (A) A graphical overview of the genomic configuration at the marker (pyrG) and target (acuB) loci in the five strains obtained during the complete procedure for two-step gene targeting with a transient marker. Strain 1 is the wild-type A. niger strain. Strain 2 is obtained after transient disruption of pyrG by the hph gene. Strain 3 is the result of the first step of a two-step gene-targeting process. In this study, acuB was replaced by the N. crassa pyr-4 deletion cassette. In this cassette pyr-4 is flanked by a DR. Strain 4 was obtained after the second step of the gene-targeting process. pyr-4 was excised, leaving a single DR sequence. At this stage, the endogenous pyrG is still disrupted and further gene targeting may be performed. When all desired gene targeting was complete, in this example only the deletion of acuB, pyrG was restored to produce strain 5, as shown in Fig. 1. For all strains, the DNA fragments liberated by XbaI and KpnI digests are indicated. The positions of the probes used in the Southern blot analysis, prb1 for pyrG and prb2 for acuB, are indicated by black boxes. For deletion of acuB, the gene-targeting substrate contained up- and downstream sequences matching the regions that are indicated by the gray “UP” and “DOWN” boxes (see Materials and Methods). (B and C) Southern blot analyses of the five different strains described above. As shown in panel B, the pyrG locus was detected by the probe prb1; as shown in panel C, the acuB loci was detected by probe prb2. Small arrows indicate relevant fragment sizes.
by serial stab inoculation on appropriate media and were shown to have lost Hyg resistance and regained PyrG function while maintaining the acuBΔ phenotype.

Southern blot analysis confirms the genomic configuration of strains obtained using the transient marker system. One purified strain representative from each stage of the gene-targeting process was analyzed by Southern blot hybridization to check the genomic configuration of the two manipulated loci (Fig. 2). As predicted by analysis of the genomic sequence data, the pyrG and the acuB loci of the wild type (strain 1) were detected as single bands representing 3.9 kb and 6.0 kb DNA fragments, respectively. When the pyrGΔ, (strain 2) was analyzed the wild-type 3.9 kb pyrG fragment was found to have been eliminated and replaced by the 2.4 kb and 4.8 kb fragments resulting from correct insertion of the hph cassette at pyrG. As expected, no change at the acuB locus at this stage in the process was observed. In the next step, acuB was targeted with the pyr-4 cassette, and in agreement with this procedure, the 6.0 kb acuB fragment detected in wild-type DNA was found to be absent in this strain (strain 3). We note that the band pattern is more complicated than the single expected 1.4 kb band predicted for a simple integration event. This phenomenon was probably caused by polymerization of the substrate prior to integration, as has been described for bipartite gene-targeting substrates in a previous study (17). However, in that study, it was shown that regardless of the complexity of the DNA that entered at the targeted locus, subsequent DR recombination reduces the locus to a point where it contains a sequence representing a single copy of the repetitive sequence. In agreement with this, we observed only the predicted 3.3 kb fragment when a random DR recombinant (strain 4) obtained from strain 3 was analyzed by Southern blotting. As expected, no change occurred at the pyrG locus during gene targeting of acuB. In the final step of the process, pyrG was restored in the acuB deletion strain. In agreement with this, the wild-type 3.9 kb pyrG fragment was regenerated in this strain (strain 5).

Concluding remarks. Here we have presented a method that allows gene targeting to be performed in a prototrophic A. niger strain, but we expect that the principles of the method can be transferred to microorganisms amenable to gene targeting. The method will likely serve to reduce experimental noise resulting from ectopically integrated genes or auxotrophies that may interfere with the analysis of an experiment or impair an industrial process. We note that 5-FOA as used in the method presented here has been reported to be slightly mutagenic (28, 29), and we therefore recommend that at least three individual strains are analyzed to confirm the phenotype resulting from the genetic manipulations.

Gene targeting for most wild-type fungi is generally cumbersome. In order to achieve the full potential of the method presented here, it may be useful to combine it with a mutation that abolishes nonhomologous end-joining activity to allow efficient gene targeting (11, 13, 15, 18, 26). We have recently constructed an A. nidulans strain with a transient disruption of this DNA repair activity (16). Hence, by combining this system with a transiently disrupted marker as presented here it will be possible to efficiently perform gene targeting in a prototrophic strain.

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REFERENCES


