Development of an Unmarked Gene Deletion System for the Filamentous Fungi *Aspergillus niger* and *Talaromyces versatilis*

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In this article, we present a method to delete genes in filamentous fungi that allows recycling of the selection marker and is efficient in a nonhomologous end-joining (NHEJ)-proficient strain. We exemplify the approach by deletion of the gene encoding the transcriptional regulator XlnR in the fungus *Aspergillus niger*. To show the efficiency and advantages of the method, we deleted 8 other genes and constructed a double mutant in this species. Moreover, we showed that the same principle also functions in a different genus of filamentous fungus (*Talaromyces versatilis*, basionym *Penicillium funiculosum*). This technique will increase the versatility of the toolboxes for genome manipulation of model and industrially relevant fungi.

Genetic studies in filamentous fungi can be limited by the tools available for genome manipulation. For example, gene replacement in *Aspergillus niger*, a filamentous fungus used in many industrial processes (1–3), is typically based on transformation with a linear DNA fragment of selection marker flanked by target gene flanking regions. This is limited by the number of selection markers available and the requirement for fungal strains with multiple auxotrophies. Moreover, DNA integration through homologous recombination is inefficient. Most integration occurs ectopically through nonhomologous end joining (NHEJ) (4, 5). To circumvent this, NHEJ-inactivated strains are used (4–7), although such strains may show genomic instability (5, 7).

We present a method for gene deletion in filamentous fungi based on recombination between a plasmid and the chromosome initially developed for *Saccharomyces cerevisiae* (8). We use *pyrG*, encoding orotidine-5-phosphate decarboxylase, as a marker that can be selected and counterselected, allowing recycling of selection markers, hence leaving no mark on the genome (9). Indeed cells lacking this enzyme cannot grow without exogenous uridine/ uracil, while these cells are resistant to the toxicity of 5-fluoro-orotic acid. To demonstrate the advantages and efficiency of this method in filamentous fungi, we deleted the *xlnR* gene coding for the xylanolytic transcriptional regulator in two species of filamentous fungi from different genera (*A. niger* and *Talaromyces versatilis*). Furthermore, 8 other genes were deleted from *A. niger*, and two genes coding for hydrophobins were deleted from the same strain. All of these gene deletions, in both strains, were performed in a strain proficient in NHEJ.

MATERIALS AND METHODS

Strains and growth media. *Escherichia coli* strain XL1-Blue MRF′ (Stratagene) was grown according to the supplier's instructions, in medium supplemented with ampicillin (100 μg/ml), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside) (30 μg/ml), and IPTG (isopropyl-β-D-thiogalactopyranoside) (1 mg/ml) (Sigma), where required, and was used for all DNA manipulation steps. The *A. niger* strain used was AB4.1 (Δ*pyrG*) (10). *A. niger* culture and medium composition were described in reference 11. The *T. versatilis* (Δ*pyrG*) strain used in this study originated from the industrial strain IMI378536, an Adisseo France S.A.S. property strain (18). This strain was maintained on potato dextrose agar (PDA) supplemented with 10 mM uridine. *T. versatilis* Δ*pyrG* cultures

were grown in 200 ml of MN-Uri medium (NaNO₃, 150 g/liter; KCl, 13 g/liter; KH₂PO₄, 38 g/liter; ZnSO₄ · 7H₂O, 22 g/liter; H₃BO₃, 11 g/liter; MnCl₂ · 4H₂O, 5 g/liter; FeSO₄ · 7H₂O, 5 g/liter; CoCl₂ · 5H₂O, 1.7 g/liter; CuSO₄ · 5H₂O, 1.6 g/liter; Na₂MoO₄ · 2H₂O, 1.5 g/liter; EDTA Na₂, 50 g/liter; glucose monohydrate, 15 g/liter; Casamino Acids, 1.25 g/liter; uridine, 2.43 g/liter; MgSO₄, 0.24 g/liter).

Construction of plasmids. Molecular cloning techniques were performed using standard procedures (12). DNA polymerase was Phusion (NEB). Primers (see the supplemental material) were designed using the *A. niger* CBS 513.88 (13) and *T. versatilis* sequences (unpublished data).

Transformation procedures. Polyethylene glycol (PEG)-mediated transformation of protoplasts was used with both fungal species using minor modifications to standard procedures (14). For both fungi, 25 mg/ml of lysing enzymes from *Trichoderma harzianum* (Sigma) were used per gram of wet mycelia. For *A. niger*, 4 mg/ml chitinase (Sigma) and 100 mg/ml bovine serum albumin (BSA) were added per gram of wet mycelium. For transformation of *A. niger*, 1.5×10^6 protoplasts were transformed with 3 μg of plasmid DNA. The transformed cell pellet was then resuspended in 300 μl STC (1.2 M sorbitol, 10 mM Tris base, 50 mM CaCl₂ [pH 7.5]), and transformed mycelia were regenerated on osmotically stabilized medium containing 1.2 M sorbitol. For transformation of *T. versatilis*, 10^7 protoplasts were transformed with 10 μg plasmid DNA, and mycelia were regenerated using procedures described elsewhere (15).

Electroporation was used to transform *E. coli*.

RESULTS AND DISCUSSION

Construction of pC3 and pC7 integrative plasmids. A 2,865-bp HindIII/EcoRI digest DNA fragment of plasmid pAo4-13 (16), containing the *pyrG* locus of *Aspergillus oryzae*, which include the
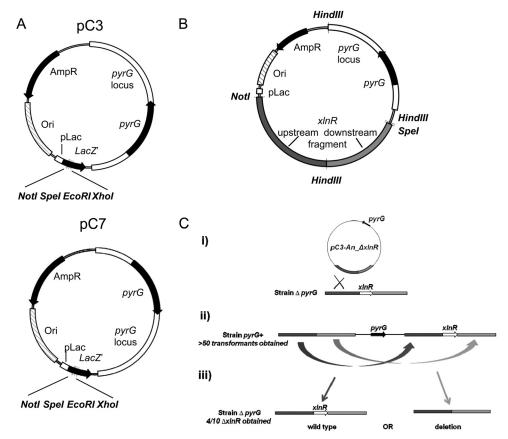
FIG 1 Plasmid vectors pC3 and PC7 and the *xlnR* deletion strategy in *Aspergillus niger*. (A) Deletion vectors pC3 and pC7. Restriction enzyme sites available in the multiple-cloning site and component parts of the vectors are indicated. The white box represents the *A. oryzae pyrG* locus obtained from plasmid pAo4-13, and the black arrow inside the *pyrG* open reading frame. (B) pC3-*An_ΔxlnR* plasmid. Flanking regions of *xlnR* are shaded: upstream in dark gray and downstream in light gray. (C) Scheme for deletion of *xlnR* in *A. niger* by the intermediate of pC3-*An_ΔxlnR* plasmid. (i) Recombination between the plasmid and the chromosome using the homology of the flanking region of *xlnR* present on the chromosome and the plasmid lead to the integration of the plasmid on the chromosome. Transformants that have integrated the plasmid are *pyrG*+ and can grow on medium lacking uridine. Transformation with pC3-*An_ΔxlnR* gave 60 to 100 *pyrG*+ transformants. (ii) Releasing of the selective pressure for plasmid integration by growing cells on a medium containing uridine allows the excision of the plasmid. When the plasmid is excised and cured, the cells are Δ*pyrG* and can be selected by plating on a medium containing uridine and 5-fluoro-orotic acid. (iii) Excision using the homology of the flanking region can lead either to reversion to the wild-type copy of the gene or to deletion. For the *xlnR* gene, 4 out of 10 clones tested by PCR were shown to be deleted.

gene and its native promoter, was blunt ended and cloned into the backbone of the plasmid pBluescript SK⁻ (Stratagene) at the PsiI site, in both orientations, generating plasmids pC3 and pC7 (Fig. 1A). We constructed plasmids with both orientations of *pyrG* in case expression of the gene interfered with insertion or excision of the plasmid during the deletion steps. The plasmids retain the blue/white screening capability for cloning and 4 unique restriction sites (EcoRI, NotI, SpeI, and XhoI) in the multiple-cloning site (MCS) to clone the flanking sequences of the locus to be deleted. These plasmids are nonreplicative in filamentous fungi as they lack an origin of replication.

Construction of the *Aspergillus niger xlnR* deletion mutant. To delete the xylanolytic transcriptional regulator-encoding gene *xlnR* in *A. niger*, 2,490-bp upstream and 1,878-bp downstream DNA fragments were amplified by PCR from *A. niger* N402 genomic DNA. Primers were designed so the upstream and downstream fragments contained a common HindIII restriction site to ligate them together and NotI and SpeI restriction sites for cloning the joined fragments into the plasmid MCS of pC3 and pC7, to create the pC3-*An_ΔxlnR* and pC7-*An_ΔxlnR* integrative plas-

mids (Fig. 1B). Transformations of the *A. niger* AB4.1 (Δ*pyrG*) strain were performed using pC3-*An_ΔxlnR* or pC7-*An_ΔxlnR* integrative plasmids. Transformations, done in triplicate, produced between 60 and 100 recombinants each, when plated on medium lacking uridine, to select for the integration of the plasmid carrying *pyrG* on the chromosome (Fig. 1C). No difference in efficiencies of transformation was observed between plasmids, indicating that there was no interference by the orientation of *pyrG* in the integration at the *xlnR* locus. Transformants were purified by propagating them twice successively on the same transformation medium but lacking sorbitol. Transformants were then propagated twice on PDA medium containing 10 mM uridine to release the selective pressure on the integrated plasmid. To select for clones that had excised the plasmid (Δ*pyrG*), spores were then resuspended in 0.001% (vol/vol) Tween 80 and spread on *Aspergillus* minimal medium containing 1% (wt/vol) glucose, 1.6 mM uridine, and 750 μg/ml 5-fluoro-orotic acid. The frequency of 5-fluoro-orotic acid-resistant strain was 8×10^{-4}.

Excision can lead to reversal to the wild-type (WT) locus or deletion of the target locus (Fig. 1C). Ten candidates were

TABLE 1 List of selected genes deleted from *A. niger* in this study[a]

Gene identification no. (CBS 513.88)	CADRE[b] annotation	Sizes of flanking regions cloned (bp)
An04g08600	Transcriptional activator XlnR	2,490 and 1,878
An02g03830	Carbon catabolite repressor CreA	2,517 and 2,000
An08g09880	Hydrophobin HfbD	1,937 and 1,476
An07g03340	Hydrophobin Hyp1	1,931 and 1,608
An09g00840	Similarity to HsbA (*Aspergillus oryzae*)	1,770 and 1,561
An18g02730	Similarity to PTH11 (*Magnaporthe grisea*)	2,036 and 1,556
An07g09330	1,4-β-D-Glucan cellobiohydrolase CbhA	1,908 and 1,727
An01g11660	1,4-β-D-Glucan cellobiohydrolase CbhB	1,960 and 1,519
An12g04610	Copper-dependent lytic polysaccharide monooxygenase	2,070 and 1,519

[a] Other deletions have also been made, including double deletions (e.g., *hfbD hyp1*).
[b] Central *Aspergillus* Data REpository.

screened, using PCR with a primer chosen to amplify the *xlnR* region. Four clones showed a product size expected for *xlnR* deletion (1,020 bp for the deletion mutant compared to 3,760 bp for the wild type) (see the supplemental material). Deletion was confirmed by sequencing the PCR product and the absence of product in another PCR with primers internal to the *xlnR* gene (see the supplemental material). All of these results confirmed that the *xlnR* gene was deleted in the *A. niger* AB4.1 strain. To check if extra copies of the plasmid remained integrated into the genome of *A. niger* due to multiple insertion of the plasmid in the chromosome of *A. niger* after the transformation, a Southern analysis of the genomic DNA restricted with BglII from the wild-type strain and the *xlnR*-deleted strain of *A. niger* was performed using pC3-An_Δ*xlnR* as a probe. The results showed no other bands than the expected 7-kbp band for the wild type and the 4.1-kbp band for the *xlnR* deletion mutant, excluding the possibility of the presence of supplementary copies of the plasmid in the genome of the *xlnR*-deleted strain (see the supplemental material).

Construction of *A. niger* single and double mutants. To show the general applicability of the method, we deleted 8 other *A. niger* genes coding for various type of proteins; transcription factor, membrane protein, plant cell wall-degrading enzymes, and small hydrophobic proteins (Table 1). All of these deletions have been confirmed by two independent PCRs, one using primers external to the gene of interest (and sequencing of the PCR products) and a second PCR using primers internal to the gene. It is of note that while one of the flanking sequences of *xlnR* cloned into the integrating plasmid was over 2 kbp (2,490 bp for the upstream fragment), successful gene deletions in *A. niger* were obtained with shorter flanking regions of the targeted gene, which can facilitate construction of the plasmid. For example, we used about 2 kbp upstream and 1.5 kbp downstream of the *hfbD* gene and about 1.8 kbp upstream and 1.6 kbp downstream of the *hsbA* gene to obtain deletion mutants (Table 1). Finally, as this approach allows for recycling of the selection marker (*pyrG*), we also created a strain carrying deletion of both hydrophobin-encoding genes *hfbD* and *hyp1*. The double mutant strain obtained by this approach was still Δ*pyrG*, so further gene deletions are still possible in this strain, allowing for dissection of complex genetic networks.

Gene deletion in *T. versatilis*. To check if the method could be extended to other fungi, deletion of the *xlnR* gene of *T. versatilis* using the pC3 integrative plasmid was performed. *T. versatilis* is an important industrial fungal species used, for example, to produce a mixture of glycosyl hydrolase enzymes. To delete the *T. versatilis xlnR* gene, 1,791-bp upstream and 1,613-bp downstream DNA

fragments of the gene were amplified from genomic DNA, ligated, and cloned in pC3 to give pC3-Tv_Δ*xlnR*. Transformation of this plasmid into a *pyrG*-deleted *T. versatilis* strain was done in duplicate, and plating on a medium lacking uracil gave 20 recombinants and 25 recombinants. This indicates that there is a functional complementation of the *T. versatilis pyrG*-deleted strain by the *A. oryzae pyrG* gene. After release of the selective pressure for plasmid integration and selection for excision of the plasmid, 9 colonies were screened for the deletion of *xlnR* by PCR using primers external to the gene. Five clones showed the expected 673-bp band corresponding to the deletion of the locus, compared to 3,470 bp of the wild-type locus (see the supplemental material). This result indicates that the method can be extended to other genera of fungi.

Conclusions. Genetic analysis in filamentous fungi can be impaired by the lack of selection markers for gene deletion and the low efficiency of homologous recombination compared to ectopic integration of linear DNA fragments. In this study, we developed a method for gene deletion that worked in two filamentous fungal species, *A. niger* and *T. versatilis*. This method should also be effective with other species and genera of filamentous fungi, providing the availability of a *pyrG* deletion strain and a functional complementation of the strain by the *pyrG* present on the plasmid. This approach is effective in more genetically stable NHEJ-proficient strains, and as it is based on recycling of the selection marker, it allows the construction of strains with multiple gene deletions. We did not examine any potential pitfalls of repeated use of selection based on the use of 5-fluoro-orotic acid, but we are aware that repeated use of 5-fluoro-orotic acid may cause chromosome alterations (16). Although it has not been directly examined here, the approach could be made more amenable for high-throughput approaches, such as the Clontech In-Fusion cloning method or, with modifications, the Life Technologies Gateway system. The method described will increase the toolbox for genome manipulation in these important and industrially relevant organisms and adds to those transformation procedures already described (2, 17).

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