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-fluoroorotic 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 (*Aspergillus 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.

**RESULTS AND DISCUSSION**

**Construction of pC3 and pC7 integrative plasmids.** A 2,865-bp HindIII/EcoRI digest DNA fragment of plasmid PA04-13 (16), containing the *pyrG* locus of *Aspergillus oryzae*, which include the
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_xlnR and pC7-An_xlnR integrative plasmids (Fig. 1B). Transformations of the A. niger AB4.1 (∆pyrG) strain were performed using pC3-An_xlnR or pC7-An_xlnR integrative plasmids. Transformations, done in triplicate, produced between 60 and 100 recombinants each, when plated on a 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 (∆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 $8 \times 10^{-4}$.

Excision can lead to reversal to the wild-type (WT) locus or deletion of the target locus (Fig. 1C). Ten candidates were...
 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 after the transformation, a Southern analysis of the genomic DNA restricted with BglII from the wild-type strain and A. niger genomic DNA from the deletion strain and a functional complementation of the xlnR-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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**REFERENCES.**


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

<table>
<thead>
<tr>
<th>Gene identification no. (CBS 513.88)</th>
<th>CADRI annotation</th>
<th>Sizes of flanking regions cloned (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>An04g06000</td>
<td>Transcriptional activator XlnR</td>
<td>2,490 and 1,878</td>
</tr>
<tr>
<td>An02g03830</td>
<td>Carbon catabolite repressor CreA</td>
<td>2,517 and 2,000</td>
</tr>
<tr>
<td>An08g09880</td>
<td>Hydropophin HfbD</td>
<td>1,937 and 1,476</td>
</tr>
<tr>
<td>An07g03430</td>
<td>Hydropophin Hyp1</td>
<td>1,931 and 1,608</td>
</tr>
<tr>
<td>An09g00840</td>
<td>Similarity to HsbA (<em>Aspergillus oryzae</em>)</td>
<td>1,770 and 1,561</td>
</tr>
<tr>
<td>An18g02730</td>
<td>Similarity to PTH11 (<em>Magnaporthe grisea</em>)</td>
<td>2,036 and 1,556</td>
</tr>
<tr>
<td>An07g03330</td>
<td>1,4-β-D-Glucan cellobiohydrolase CBHA</td>
<td>1,908 and 1,727</td>
</tr>
<tr>
<td>An10g16660</td>
<td>Similarity to PTH11 (<em>Magnaporthe grisea</em>)</td>
<td>1,908 and 1,727</td>
</tr>
<tr>
<td>An12g04610</td>
<td>Copper-dependent lytic polysaccharide monooxygenase</td>
<td>2,070 and 1,519</td>
</tr>
</tbody>
</table>

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


