Application of the *Saccharomyces cerevisiae* FLP/*FRT* Recombination System in Filamentous Fungi for Marker Recycling and Construction of Knockout Strains Devoid of Heterologous Genes[▽][†]

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To overcome the limited availability of antibiotic resistance markers in filamentous fungi, we adapted the FLP/*FRT* recombination system from the yeast *Saccharomyces cerevisiae* for marker recycling. We tested this system in the penicillin producer *Penicillium chrysogenum* using different experimental approaches. In a two-step application, we first integrated ectopically a nourseothricin resistance cassette flanked by the *FRT* sequences in direct repeat orientation (*FRT-nat1* cassette) into a *P. chrysogenum* recipient. In the second step, the gene for the native yeast FLP recombinase, and in parallel, a codon-optimized *P. chrysogenum flp* (*Pcflp*) recombinase gene, were transferred into the *P. chrysogenum* strain carrying the *FRT-nat1* cassette. The corresponding transformants were analyzed by PCR, growth tests, and sequencing to verify successful recombination events. Our analysis of several single- and multicopy transformants showed that only when the codon-optimized recombinase was present could a fully functional recombination system be generated in *P. chrysogenum*. As a proof of application of this system, we constructed a ΔPcku70 knockout strain devoid of any heterologous genes. To further improve the FLP/*FRT* system, we produced a flipper cassette carrying the *FRT* sites as well as the *Pcflp* gene together with a resistance marker. This cassette allows the controlled expression of the recombinase gene for one-step marker excision. Moreover, the applicability of the optimized FLP/*FRT* recombination system in other fungi was further demonstrated by marker recycling in the ascomycete *Sordaria macrospora*. Here, we discuss the application of the optimized FLP/*FRT* recombination system as a molecular tool for the genetic manipulation of filamentous fungi.

Site-specific recombination is an important molecular tool for functional genetic studies in both prokaryotes and eukaryotes and is mediated by two major recombinase families, the resolvase/invertase family and the integrase family (9, 65). The resolvase/invertase family is characterized by the conserved catalytic amino acid serine, which allows intramolecular reactions, whereas the integrase family is able to mediate both intra- and intermolecular recombinations due to an autocatalytic activity of the conserved residue tyrosine (19, 35). The best-studied members of the integrase family are the λ integrase of bacteriophage λ, the recombinase Cre of *Escherichia coli* bacteriophage P1 (1, 62), the XerCD proteins of *E. coli* (6), and the eukaryotic FLP recombinase of the yeast *Saccharomyces cerevisiae* (9, 51). Common to all these systems are two different or identical recognition sites that serve as the DNA substrate for the recombinase.

In recent years, recombinases have become important tools to manipulate genetically prokaryotes as well as eukaryotes. In this study, we have modified the FLP/*FRT* recombination system from yeast for application in filamentous fungi. The FLP/*FRT* recombination system is encoded by the 2μm (6.4 kb) plasmid that is present in most isolates of *S. cerevisiae*. Due to

its stable partitioning and amplification system, this plasmid is available with a copy number of 60 to 100 copies per cell (16, 63). If the copy number drops below a certain level, the 45-kDa FLP recombinase catalyzes recombination between two 599-bp sites. These sequences are present in inverted orientation and allow the 2μm plasmid to exist in two conformations, namely, the A conformation when it is silent and the B conformation when it can replicate itself and thus maintain a constant plasmid copy number per cell (64). Analysis of the 599-bp inverted repeat sequence revealed that only 34 bp, the FLP recognition targets (*FRT*), are necessary for successful recombination (54). For every FLP-mediated recombination, a total of four FLP recombinases and two *FRT* sequences are needed. Two of the four proteins bind to one *FRT* sequence because every *FRT* has two 13-bp FLP-binding sites which are interrupted by an 8-bp spacer region. In this spacer DNA strand breakage takes place, producing 8-bp overhanging ends. After strand breakage, the overhanging ends of the two *FRT* fragments come together by complementary base pairing so that a recombinant *FRT* sequence is generated (2, 51).

The FLP/*FRT* recombination system has been successfully applied in model organisms such as *E. coli* (10, 55) as well as in pathogenic and nonpathogenic Gram-negative and Gram-positive bacteria (53). In addition to prokaryotes, the FLP/*FRT* recombination system has been adapted for use in several eukaryotes (26). For example, it is well established in diverse yeasts (11, 41, 57, 60) and has been used successfully to modify the genomes of higher eukaryotes, including animal and human cell lines (5, 37, 58). Despite this tool's application in

TABLE 1. Fungal strains used in this study

Organism	Strain	Genotype	Reference
P. chrysogenum	P2niaDi8	Penicillin producer; *niaD⁻*	23
	ΔPcku70	*ΔPcku70::trpC(p)::nat1*	22
	PcFRT1	*FRT::trpC(p)::nat1::FRT*	This study
	PcFRT2Y	*FRT::trpC(p)::nat1::FRT trpC(p)::flp ptrA(p)::ptrA*	This study
	PcFRT2P	*FRT trpC(p)::Pcflp ptrA(p)::ptrA*	This study
	ΔPcku70FRT1	*ΔPcku70::FRT::trpC(p)::ble::FRT*	This study
	ΔPcku70FRT2	*trpC(p)::Pcflp ptrA(p)::ptrA ΔPcku70::FRT*	This study
	PcFLIP1	*FRT::xyl(p)::Pcflp::trpC(p)::nat1::FRT*	This study
	PcFLIP2	*FRT*	This study
S. macrospora	SmFRT1	*FRT::trpC(p)::nat1::FRT trpC(p)::Pcflp trpC(p)::hph*	This study
	SmFRT2P	*FRT trpC(p)::Pcflp trpC(p)::hph*	This study

higher eukaryotes, it has not yet been used in filamentous fungi. We present the first application of the FLP/*FRT* recombination system in a filamentous fungus, the major penicillin producer *Penicillium chrysogenum*. A limiting factor to manipulate *P. chrysogenum* genetically is the small number of suitable resistance markers. To overcome this problem, we generated a codon-optimized FLP/*FRT* recombination system to establish an efficient marker recycling system in this fungus. Using the optimized FLP/*FRT* recombination system, we demonstrate in a two-step approach the functionality of this system by construction of a marker-free *P. chrysogenum* strain lacking the *ku70* (*Pcku70*) gene for optimized homologous recombination. A further advancement was the construction of a novel inducible *nat1* flipper cassette allowing marker recycling in a one-step approach. The broader applicability of this recombination system was finally shown by its use in the model ascomycete *Sordaria macrospora* (34).

MATERIALS AND METHODS

Strains, culture conditions, and transformation procedure. *E. coli* strain K-12 XL1-blue (Stratagene) was used for plasmid construction and maintenance (7). All *P. chrysogenum* and *S. macrospora* strains used in this study are listed in Table 1. *P. chrysogenum* P2niaD18 and the ΔPcku70 strain lacking the *Pcku70* gene (22) as well as the *S. macrospora* wild-type strain (34) served as recipients for the integration of different *FRT*-flanked resistance cassettes.

All *P. chrysogenum* strains were grown at 27°C and 120 rpm for 3 days in liquid complex culture medium (CCM) or minimal medium (MM) or grown on solid CCM or MM (21, 39). To induce the *P. chrysogenum* flp (Pcflp)-mediated site-specific recombination, appropriate strains were grown on solid MM supplemented with 2% xylose (25). Liquid or solid Burkholder's minimal medium (BMM) was used for the cultivation of the *S. macrospora* strains (45).

Transformation of individual *P. chrysogenum* strains was performed as described by Janus et al. (25) and Hoff et al. (22). Transformation of *S. macrospora* was preformed according to Nowrousian et al. (45). Transformants were selected by growth on medium containing 150 μg ml⁻¹ nourseothricin, 40 μg ml⁻¹ phleomycin, or 7 μg ml⁻¹ pyrithiamine.

Growth tests. Recombination events in transgenic strains were detected by growth tests on MM and CCM containing 200 μg ml⁻¹ nourseothricin, 40 μg ml⁻¹ phleomycin, or 7 μg ml⁻¹ pyrithiamine. Strains were grown for 5 days at 27°C. All transformants and the wild-type strains were grown on CCM without selection, as positive controls.

Construction of recombinant plasmids. For construction of the *FRT-nat1* cassette, the *FRT* sequences of the native FLP/*FRT* recombination system were used to flank the nourseothricin resistance gene (*nat1*) of *Streptomyces noursei* at the 5′ and 3′ ends. For this purpose the complementary oligonucleotides FRT1 and FRT2 were annealed to obtain the 5′ *FRT* sequence, and the primers FRT3 and FRT4 were used to generate the 3′ *FRT* sequence. The 5′ *FRT* was first inserted between the PstI and BamHI restriction sites of plasmid pD-NAT1 (32), generating plasmid pDNAT1-FRT1. This plasmid was then hydrolyzed with SacI

and NotI and used for ligation with the 3′ *FRT*. The resulting plasmid carries the full *FRT-nat1* cassette and was named pDNAT1-FRT1-2.

In the case of plasmid pKOKU70_Phleo, a 5′ *ku70-FRT* fragment was amplified with primers 5′ku70FRT_s and 5′ku70FRT_a, and PCR with pKOKU70 as a template was conducted as published by Hoff et al. (22). The resulting 1.1-kb amplicon was ligated into plasmid pDrive (Qiagen, Hilden, Germany), and DNA sequencing was done by custom-provided services (MWG-Biotech, Germany). Plasmid pD5ku70FRT was hydrolyzed with KpnI-BamHI and the 5′ *ku70-FRT* fragment was ligated into plasmid pD-Phleo (22) using the corresponding restriction sites. The resulting recombinant plasmid was named p5ku70FRT_Phleo. In the next step, oligonucleotides 3′ku70FRT_s and 3′ku70FRT_a were annealed, and the resulting 3′ *FRT* site was ligated into p5ku70FRT_Phleo using EcoRI-HindIII restrictions sites to generate plasmid p53ku70FRT_Phleo. In a final step, plasmid pKOKU70 was hydrolyzed with NotI and HindIII, and the obtained 3′ *ku70* fragment was ligated in p53ku70FRT_Phleo using the corresponding restriction sites. The resulting plasmid was designated pKOKU70_Phleo and carries the complete *FRT-ku70* cassette together with the integrated *ble* gene under the control of the *trpC* promoter [*trpC(p)*] from *Aspergillus nidulans*.

For construction of plasmid pPTRII_FLP the recombinase gene was amplified with the primers 5-FLP and 3-FLP from plasmid pSFU1 (40) and further subcloned into pDrive (Qiagen, Germany) for DNA sequence analysis. In the resulting recombinant plasmid, the *flp* gene was flanked by the NcoI and SalI sites, which were used in a subsequent step to integrate *flp* behind the strong *trpC* promoter of plasmid pD-Phleo. In this plasmid a 1.7-kb KpnI fragment carries the fungal *trpC* promoter upstream of the *flp* gene. In order to generate a fungal transformation vector the 1.7-kb fragment was inserted into the free-replicating plasmid pPTRII (31). This new vector was named pPTRII_FLP.

The optimized *Pcflp* recombinase gene was cloned into plasmid pUC57PcFLP (GenScript). The sequence was further modified by amplification with primers P3, P4, and pUC57PcFLP as templates. Since primer P4 contains recognition sequences for KpnI and HindIII, the recombinant *Pcflp* sequence was flanked by these restriction sites. The resulting *Pcflp* gene, with a size of 1.3 kb, was ligated into pDrive, sequenced (MWG-Biotech, Germany), and subsequently isolated using NcoI-HindIII restriction sites. This fragment was ligated into plasmid pD-Phleo using restriction sites NcoI-HindIII. The resulting recombinant plasmid, carrying the *Pcflp* gene under the control of the *trpC* promoter, *trpC(p)*, from *A. nidulans*, was designated pPtrpCPcFLP. In a final step, the *trpC(p)-Pcflp* fragment was digested with KpnI and cloned into the unique restriction site of plasmid pPTRII to generate plasmid pPTRII_PcFLP.

Plasmid pGPS2.1-Pcflp was designed as described by Dreyer et al. (12) with the following modifications: plasmid pPtrpCPcFLP was hydrolyzed with KpnI to isolate the *Pcflp* gene under the control of the *trpC* promoter, which was then integrated into pGPS2.1-hph.

The *nat1* flipper cassette was designed by first annealing the complementary oligonucleotides 5FRTlong_s and 5FRTlong_a as well as 3FRTlong_s and 3FRTlong_a to produce either the 5′ *FRT* or 3′ *FRT* sequence. The 3′ *FRT* fragment was introduced into the NotI-ApaI restriction sites of plasmid pTOPO (Invitrogen, Germany). The resulting plasmid p3FRT was digested using HindIII and SacI for insertion of the 5′ *FRT* site to produce p53FRT. The next step involved eluting a 1.0-kb cassette containing the *nat1* marker gene under the control of the *trpC* promoter from plasmid pD-NAT1 and inserting it into the BamHI-SacI restriction sites of plasmid p53FRT to generate p53FRTnat. Finally, p53FRTnat was hydrolyzed with NotI and BamHI to integrate the 3.0-kb

TABLE 2. Sequences of oligonucleotides used in this work

Oligonucleotide	Sequence (5′–3′)	Specificity[a]
FRT1	GGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCG	5′ FRT sense + PstI + BamHI
FRT2	GATCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCCTGCA	5′ FRT antisense + PstI + BamHI
FRT3	CGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGC	3′ FRT sense + SacI + NotI
FRT4	GGCCGCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCGAGCT	3′ FRT antisense + SacI + NotI
5FRTlong_s	AGCTTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGAGCT	5′ FRT sense + HindIII + SacI
5FRTlong_a	CGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCA	5′ FRT antisense + HindIII + SacI
3FRTlong_s	GGCCGCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGGCC	3′ FRT sense NotI + ApaI
3FRTlong_a	CGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCG	3′ FRT antisense NotI + ApaI
5′ku70FRT_s	GGTACCATCCTCCATTTGCGCGCTTTCCCT	pKOKU70 (nt 285–308) + KpnI
5′ku70FRT_a	GGATCCGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCTGTTAGCAAGTAGGTATCCTCGGGAGATTGGAAATATTAAAAGGTGTAAA	pKOKU70 (nt 1256–1305) + BamHI
3′ku70FRT_s	AATTCGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCA	3′ FRT sense + EcoRI + HindIII
3′ku70FRT_a	AGCTTGAAGTTCCTATTCTCTAGAAAGTATAGGAACTTCG	3′ FRT antisense + EcoRI + HindIII
5-FLP	CTCCATGGGAATGCCACAATTTGGTATATTATGTAA	flp gene of plasmid pSFU1 (nt 1–26) + NcoI
3-FLP	GAGTCGACATAGGTACCTTATATGCGTCTATTTATGTAGGATGAAAGGT	flp gene of plasmid pSFU1 (nt 1250–1281) + KpnI + SalI
P1	GCGGGCAGTGAGCGCAACGCAATTAA	pXPcFLPnat1FRT (nt 84–109)
P2	GGCCTCTTCGCTATTACGCCAGCT	pXPcFLPnat1FRT (nt 4444–4467)
P3	ATCTAGATACCATGGGTATGCCCCAGTT	pUC57PcFLP (nt 424–451)
P4	TAAAGCTTAATGGTACCTAGGATCCTTAGATGCGGCGGT	pUC57PcFLP (nt 1699–1720) + KpnI + HindIII
P5	CTCCTCTGGGTAGGTCTTAAGCTG	5′ flanking region of Pcku70 (22)
P8	CCCCCAGGTGGCCAAAGCATCTT	3′ flanking region of Pcku70 (22)
FN_s	CCCAGGCTTTACACTTTATGCTT	pNATFRT1-2 (nt 135–157)
FN_a	CCAGTGAATTGTGCGGCCATTT	pNATFRT1-2 (nt 1420–1441)
recombinant_FRT	GTGAGCGGATAACAATTTCA	Recombinant FRT sequence (nt 182–201)

[a] nt, nucleotide.

xylP-Pcflp construct; the resulting plasmid was named pXPcFLPnatFRT and contained the complete *nat1* flipper cassette. The sequences of all oligonucleotides and features of recombinant plasmids are given in Tables 2 and 3.

Molecular analysis of fungal transformants. Genomic DNA from both primary and secondary transformants was isolated as described previously (22, 48). For molecular analysis, genomic DNA of transformants from strain PcFRT1 was hydrolyzed with BamHI, separated by agarose gel electrophoresis, and blotted for Southern hybridization (52). A ^{32}P-radiolabeled, 0.9-kb *nat1* fragment was chosen as the probe. Bulk DNA was used for PCR analysis (23), followed by agarose gel electrophoresis to separate the amplicons. Template DNA from primary transformants or plasmid pDNAT1-FRT1-2 served as a positive control and generated a fragment of 1.3 kb with primers FN_s and FN_a (see Fig. 2). However, both primers amplified a 0.2-kb DNA molecule when DNA from secondary transformants PcFRT2Y (PcFRT2 carrying the yeast *flp* gene), PcFRT2P (PcFRT2 carrying the *Penicillium*-optimized codon usage), and SmFRT2 (*S. macrospora* secondary transformant) was used. The 0.2-kb fragment was sequenced (MWG, Germany) and further analyzed using the program LALIGN http://www.ch.embnet.org/software/LALIGN_form .html). In the case of strain PcFLIP1, the PCR analysis using genomic DNA generated fragments with sizes of 2.5 and 3.1 kb, whereas a 0.3-kb amplicon could be detected in the PcFLIP2 strain.

Bioinformatics analysis. In order to construct a codon-optimized *flp* gene, the codon usage of *P. chrysogenum* was analyzed using databases at http://www.kazusa .or.jp/codon/ and genome sequence data from van den Berg et al. (61). To decrease the GC content in the optimized recombinase sequence, not only the optimal codon triplets were chosen but also triplets in the second or third preference. After optimal codon selection, codon optimization was verified by using the program JCat (Java Codon Adaptation Tool [http://www.jcat.de/]) (18). The adapted recombinase sequence was extended at both ends using the restriction enzymes NcoI and BamHI and sent to GenScript (Piscataway, NJ) for custom synthesis and cloning into pUC57PcFLP. The resulting recombinant gene was named *Pcflp*.

RESULTS

Construction of the *FRT-nat1* cassette and generation of primary transformants. To generate a functional FLP/*FRT* recombination system, we constructed an *FRT-nat1* cassette in which the nourseothricin resistance gene (*nat1*) of *Streptomyces noursei* is flanked by two *FRT* sites in direct-repeat orientation. In this construct, which is contained in plasmid pDNAT1_FRT1-2, the *nat1* gene encoding an *N*-acetyltransferase is under the control of the strong and constitutive fungal *trpC* promoter from *A. nidulans* (32). For fungal DNA-mediated transformation, a 1.5-kb EarI restriction fragment from pDNAT1_FRT1-2, in which the *FRT-nat1* cassette is flanked by short bacterial plasmid sequences, was used.

The ectopic integration of this linear DNA fragment into the *P. chrysogenum* recipient strain P2niaD18 generated a large number of primary transformants (PcFRT1). To confirm the presence of the *FRT-nat1* cassette in PcFRT1 transformants, oligonucleotides FN_s and FN_a were used for PCR amplification from genomic DNA. The primers hybridize to the bacterial sequences of the linear transformation fragment, thus allowing amplification of the ectopically integrated cassette that otherwise is flanked by uncharacterized genomic DNA sequences. As expected, a 1.3-kb fragment was detected in all transformants (data not shown). Six recombinant strains, designated T2, T3, T4, T11, T12, and T18, were chosen for Southern hybridization analysis. The autoradiograph in Fig. 1B obtained from hybridization with a *nat1* gene fragment as a radio-labeled probe identified a single band in strains T2 and T4, indicating that single-copy integration events of the *FRT-nat1* cassette had taken place. In contrast, the several bands found in the other lanes indicate multicopy transformants. T11, which had four hybridizing bands, was chosen for further analysis as a representative example of a multicopy *FRT-nat1*

TABLE 3. Plasmids used to establish the native and optimized FLP/*FRT* recombination system in *P. chrysogenum*

Plasmid	Characteristic(s)[a]	Reference or source
pD-NAT1	*trpC* promoter of *A. nidulans*, *nat1* gene of *S. noursei*	32
pDNAT1-FRT1	*trpC* promoter of *A. nidulans*, 5′ *FRT* sequence, *nat1* gene of *S. noursei*	This study
pDNAT1-FRT1-2	5′ *FRT* sequence, *trpC* promoter of *A. nidulans*, *nat1* gene of *S. noursei*, 3′ *FRT* sequence	This study
pKOKU70	5′ *ku70* fragment, *trpC* promoter of *A. nidulans*, *nat1* gene of *S. noursei*, 3′ *ku70* fragment	22
pD5ku70FRT	5′ *ku70* fragment	This study
p5ku70FRT_Phleo	5′ *ku70* fragment, 5′ *FRT* sequence, *trpC* promoter of *A. nidulans*, *ble* resistance gene of *S. hindustanus*	This study
p53ku70FRT_Phleo	5′ *ku70* fragment, 5′ *FRT* sequence, *trpC* promoter of *A. nidulans*, *ble* resistance gene of *S. hindustanus*, 3′ *FRT* sequence	This study
pKOKU70_Phleo	5′ *ku70* fragment, 5′ *FRT* sequence, *trpC* promoter of *A. nidulans*, *ble* resistance gene of *S. hindustanus*, 3′ *FRT* sequence, 3′ *ku70* fragment	This study
p3FRT	3′ *FRT* sequence	This study
p53FRT	5′ *FRT* sequence, 3′ *FRT* sequence	This study
p53FRTnat	5′ *FRT* sequence, *trpC* promoter of *A. nidulans*, *nat1* gene of *S. noursei*, 3′ *FRT* sequence	This study
pXPcFLPnatFRT	5′ *FRT* sequence, *xyl* promoter of *P. chrysogenum*, *Pcflp* gene, *trpC* promoter of *A. nidulans*, *nat1* gene of *S. noursei*, 3′ *FRT* sequence	This study
pSFU1	*URA3* flipper cassette	40
pDrive	UA-based PCR cloning	Qiagen, Germany
pDFLP	*flp* gene	This study
pD-Phleo	*trpC* promoter of *A. nidulans*, *ble* resistance gene of *S. hindustanus*	22
pPtrpCFLP	*trpC* promoter of *A. nidulans*, *Pcflp* gene	This study
pPTRII	Self-replicating plasmid; *ptrA* resistance gene of *A. oryzae*, *AMA1* sequences of *A. nidulans*	31
pPTRII_FLP	*trpC* promoter, *flp* gene, *ptrA* resistance gene of *A. oryzae*, *AMA1* sequences of *A. nidulans*	This study
pUC57PcFLP	*Pcflp* gene	GenScript
pPtrpCPcFLP	*trpC* promoter of *A. nidulans*, *Pcflp* gene	This study
pPTRII_PcFLP	*trpC* promoter, *Pcflp* gene, *ptrA* resistance gene of *A. oryzae*, *AMA1* sequences of *A. nidulans*	This study
pGPS2.1-hph	*trpC* promoter of *A. nidulans*, *hph* of *K. pneumoniae*, left and right borders from transposon Tn7 (New England Biolabs)	12
pGPS2.1-PcFLP	*trpC* promoter of *A. nidulans*, *hph* of *K. pneumoniae*, left and right borders from transposon Tn7 (New England Biolabs), *Pcflp* gene	This study

[a] *S. hindustanus*, *Streptoalloteichus hindustanus*; *K. pneumoniae*, *Klebsiella pneumoniae*.

transformant. Both the single- and multicopy strains were used to generate secondary transformants.

Synthesis of a codon-optimized *flp* recombinase gene for construction of an expression plasmid. When we compared the codon bias of the native *flp* recombinase gene from yeast with the general codon usage in *P. chrysogenum* using the online databases at http://www.kazusa.or.jp/codon/ and the genome sequence of *P. chrysogenum* (61), we found distinct differences. For example, in the native *flp* gene, the amino acid arginine is mostly encoded by the triplets AGA and AGG but never by codon CGC. In contrast, CGC is the favored codon for arginine in *P. chrysogenum*, with AGA and AGG being used only in <10% of cases (data not shown). Furthermore, the *S. cerevisiae flp* gene has a GC content of 37%. In contrast, the nuclear genome of *P. chrysogenum* has an overall GC content of 48.9%, with exon regions having a GC content of 52.8% and intron sequences having a lower GC content of 45.3% (61). Due to these differences between *S. cerevisiae* and *P. chrysogenum*, the codon usage bias for each amino acid in the *flp* recombinase gene was determined. Based on these properties, we decided to synthesize an *flp* recombinase gene with a bias for preferred *P. chrysogenum* codons.

In a first attempt, we generated a codon-optimized recombinase gene *in silico* with a GC content of 61%. Since *P. chrysogenum* exons have an average GC content of 52.8%, we generated *in silico* a second version with a mixture of codons used in the first, second, and third preferences. The resulting sequence had a GC content of 57% with 73% DNA homology to the native *flp* recombinase gene, despite modification of over 66% of all triplets. To determine whether the modified *Pcflp* sequence was mostly compatible for *P. chrysogenum*, we used the program JCat (18) for codon adaptation. This program compares the sequence of any gene with the codon usage database of a chosen organism. As a database for *P. chrysogenum* is not available in JCat, the database of the next most closely related filamentous fungus, *Aspergillus niger*, was used to test the quality of the codon adaptation as the species have a very similar codon usage (47, 61). The codon adaptation index (CAI) was calculated for both the native *flp* gene and the codon-optimized *Pcflp* sequence. The CAI can have a value between 0 and 1; the more the CAI tends to 1, the less optimization is needed. The native *flp* recombinase gene has a CAI of 0.17, indicating that this gene is not fully suitable for optimal expression in *P. chrysogenum*. In contrast, the CAI of 0.81 of the codon-adapted *Pcflp* recombinase gene (see Fig. S1 in the supplemental material) is a strong indicator that this gene can be used for optimal expression in filamentous fungi.

Expression of the native and codon-adapted FLP recombinase genes in *P. chrysogenum*. Using the primary PcFRT1 transformants T2, T4, and T11, we generated secondary transformants that carry either the yeast gene (*flp*) or the codon-optimized recombinase gene (*Pcflp*) by transformation with the
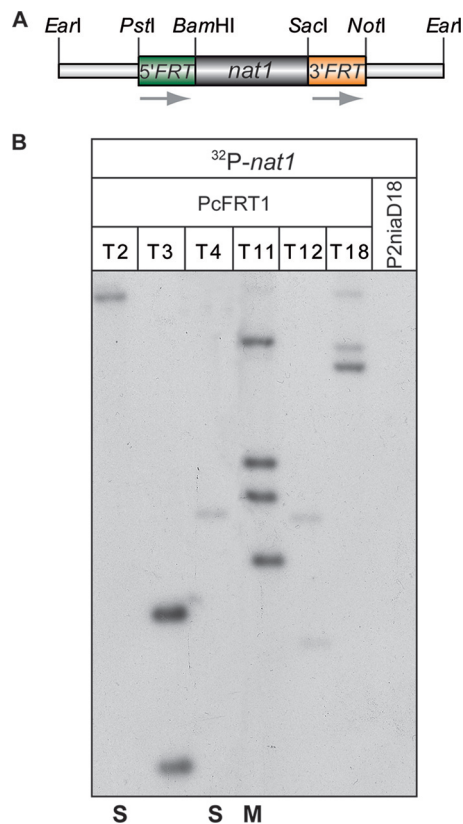
FIG. 1. Characterization of primary transformants from *P. chrysogenum.* (A) Schematic representation of the *FRT-nat1* cassette. Adjacent to the *nat1* resistance gene are two *FRT* sites in direct repeat orientation, and both are flanked by vector sequences. (B) Southern hybridization analysis of the primary PcFRT1 transformants to identify the copy number of ectopically integrated *FRT-nat1* cassettes. Genomic DNAs of the recipient strain P2niaD18 and the transformants were digested with BamHI and hybridized with a ^{32}P-labeled *nat1* probe. The single-copy transformants T2 and T4 and the multicopy transformant T11 were chosen for further experimental analyses. S, single copy; M, multicopy.

self-replicating plasmids pPTRII_FLP and pPTRII_PcFLP, respectively. The resulting transformants, carrying the yeast gene (PcFRT2Y) or the synthetic recombinase gene with the *Penicillium*-optimized codon usage (PcFRT2P), were characterized by PCR analysis, growth tests, and sequencing to verify the functionality and efficiency of the FLP/*FRT* systems in *P. chrysogenum*. We expected that after FLP- or Pcflp-mediated site-specific recombination, a single, recombinant 34-bp *FRT* site remains as a remnant of the complete *FRT-nat1* in the genome of the resulting strain. As a consequence, the secondary transformants should be sensitive to nourseothricin.

In the case of strains transformed with *flp*, growth tests showed that none of 30 secondary PcFRT2Y transformants was nourseothricin sensitive when grown on the selection medium. Growth tests with strains transformed with *flp* showed that none of 40 secondary transformants had lost the nourseothricin resistance gene. To exclude the possibility that heterokaryons are responsible for nourseothricin resistance, a total of 300 single-spore isolates from two single-copy and two multicopy PcFRT2Y transformants were tested on nourseothricin-

containing medium (data not shown). None had lost the resistance marker, indicating that only a very minor fraction of nuclei is carrying the *FRT* sequence without the resistance marker gene.

A completely different result was obtained with transformants PcFRT2P carrying Pcflp. All 34 tested transformants derived either from the single-copy PcFRT1 T2 or T4 transformant showed sensitivity to the antibiotic nourseothricin. Similarly, 16 out of 20 analyzed transformants received from the multicopy transformant PcFRT1 T11 showed no growth on nourseothricin-containing medium (see Fig. S2 in the supplemental material). These data clearly indicate that the codon-adapted FLP/*FRT* recombination system can efficiently work in different *P. chrysogenum* transformants.

These results from the growth tests correlate perfectly with our PCR analyses (Fig. 2). As indicated in Fig. 2A, oligonucleotides FN_s and FN_a hybridize to sequences flanking the *FRT-nat1* cassette. Amplification of the cassette with these two primers generated a 1.3-kb amplicon, whereas a 0.2-kb fragment was generated after excision of the *FRT-nat1* cassette by an FLP-mediated site-specific recombination event. For amplification, we used genomic DNA from the above-described fungal transformants carrying one of the two recombinase genes. All recombinant strains (PcFRT2Y) carrying the yeast *flp* gene delivered both the large and the small amplicons, thus indicating that the recombinase-mediated recombination was highly inefficient in these transformants (Fig. 2B). The large fragment of 1.3 kb, however, was completely missing in all transformants (PcFRT2P) carrying the codon-optimized *Pcflp* recombinase gene. From the results of the PCR analyses together with data from growth tests, we conclude that all *FRT-nat1* cassettes were excised from the chromosomal DNA.

That successful recombination had occurred was confirmed by sequencing a recombinant *FRT* sequence from the secondary transformants PcFRT2P. This site consists of the left and right halves from both *FRT* sequences of the *FRT-nat1* cassette (Fig. 3). Both *FRT* sequences are flanked by different restriction sites, namely, by the PstI and BamHI and by the pair SacI and NotI. After excision of the *FRT-nat1* cassette, the recombinant *FRT* should be flanked by restriction sites PstI and NotI. Sequencing of the 0.2-kb fragment from the secondary PcFRT2P transformants confirmed this prediction (Fig. 3). Taken together, our data clearly demonstrate that the optimized FLP/*FRT* recombination system is functional in *P. chrysogenum* and can be used as an efficient marker recycling system to generate multiple deletion strains with a limited number of resistant markers.

Construction of a marker-free recipient for the generation of knockout strains. We have recently reported the generation of a ΔPcku70 strain as a recipient for the construction of knockout strains in *P. chrysogenum* (22). As shown in Fig. 4A, the *Pcku70* gene of P2niaD18 was replaced in the ΔPcku70 strain by homologous recombination using the *nat1* resistance marker. This strain was used as recipient for a further transformation experiment with a vector molecule carrying the phleomycin (*ble*) resistance marker. The *ble* gene is flanked by two *FRT* sites as well as sequences homologous to the 5′ and 3′ regions of the *Pcku70* gene. After selection of transformants on phleomycin-containing medium, we obtained a

FIG. 2. Analysis of secondary transformants. (A) Schematic map of the *FRT-nat1* cassette before (top) and after (bottom) site-specific excision. Arrows indicate location of PCR primers and bars show the length of the predicted amplicons. (B) PCR analysis of secondary transformants as indicated. PcFRT2Y and PcFRT2P indicate fungal transformants carrying either the yeast (*flp*) or the codon-adapted recombinase (*Pcflp*) gene, respectively. T2.1, T2.2, T2.4, T2.5, T4.1, T4.2, T4.4, and T4.5 indicate single-copy primary transformants while T11.1, T11.2, T11.4, and T11.5 represent multicopy primary transformants. A 1.3-kb amplicon is generated when the complete *FRT-nat1* cassette is present in the tested transformants, whereas a 0.2-kb fragment could be amplified only after an excision of the *FRT-nat1* cassette by an FLP-mediated site-specific recombination event. DNA of plasmid pFRT served as a positive control.

ΔPcku70FRT1 strain where the *nat1* cassette was replaced by the *ble* marker. Due to efficient homologous recombination, 9 out of 18 tested transformants contained the expected substitution. These strains have lost the ability to grow on nourseothricin-containing medium. For the next step, we used two randomly selected transformants as recipients to introduce the self-replicating plasmid pPTRII_PcFLP carrying the *Pcflp* recombinase gene. After selection on pyrithiamine, a total of 18 transformants were tested for their capability to still grow on phleomycin. Two transformants (ΔPcku70FRT2) had lost the phleomycin resistance phenotype, thus suggesting that excision of the phleomycin resistance gene had occurred (see Fig. S3 in the supplemental material). The above described substitutions

and excisions in the diverse strains shown in Fig. 4 were further verified by PCR analyses. Amplification was performed with primer pair P5 and P8 flanking the *Pcku70* region and located outside the recombination sites (Fig. 4A). As indicated in Fig. 4B, the different events result in amplicons that can clearly be distinguished by their sizes. The smallest amplicon of 2.4 kb, indicating a Pcflp-mediated site-specific recombination event, was found in the ΔPcku70FRT2 strain. A further loss of the recombinase gene carrying the plasmid was achieved by growth of the transformants on pyrithiamine-free medium. In conclusion, we have constructed a recipient strain for efficient homologous recombination that is free of any heterologous gene sequences. Only the 34 bp of a single *FRT* site reside within the fungal genome. This experimen-

FIG. 3. Structure and sequence of a recombinant *FRT* sequence in the genomic DNA. *FRT* sequences before (top) and after (middle) recombination are flanked by different restriction sites, as indicated. The recombinant DNA sequence obtained from PcFRT2P is shown at the bottom.
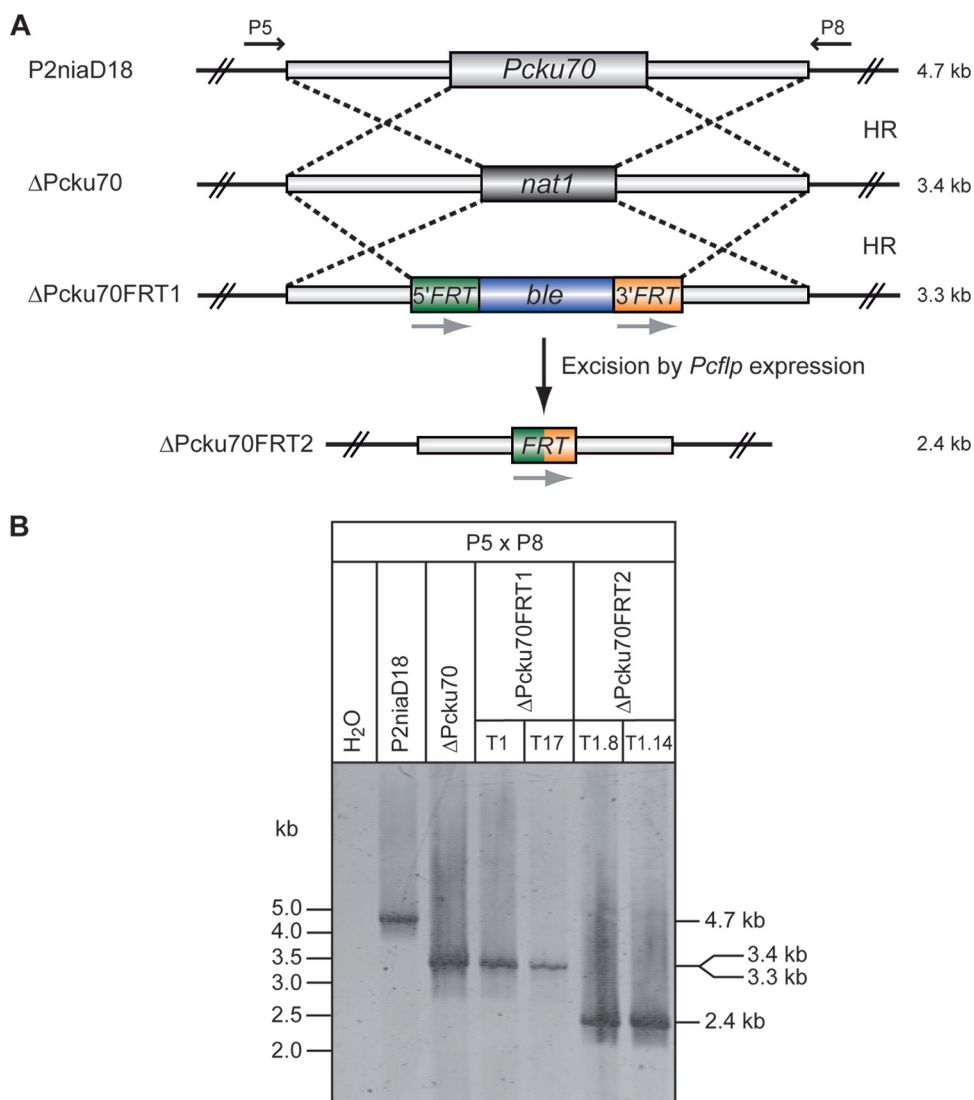
FIG. 4. Generation of a marker-free ΔPcku70 strain. (A) Strategy for construction of different strains. Location of primers for the PCR analysis in panel B is shown, and the predicted sizes of amplicons are given. (B) PCR analysis with DNA from different strains as indicated. HR, homologous recombination. P5 × P8 indicates the pair of primers used for PCR amplification.

tal approach and the generated transformants provide the basis for both the *nat1* and the *ble* marker to be used for further construction of double or triple knockout mutants.

Construction of an *nat1* flipper cassette for one-step marker excision. All above-described experiments require a two-step strategy for successful excision of the *FRT-nat1* cassette. We therefore constructed an *nat1* flipper cassette for one-step marker recycling. The cassette contains the *nat1* resistance marker as well as the gene for the Pcflp recombinase, which is under the control of the xylose-inducible *xyl* promoter (70). As shown in Fig. 5A, both genes are flanked by the two *FRT* sites on the *nat1* flipper cassette. For transformation of P2niaD18, a linear restriction fragment was used carrying the *nat1* flipper cassette as well as flanking plasmid sequences. The corresponding transformants (PcFLIP1) were selected on nourseothricin-containing medium with glucose as a carbon source. To induce the Pcflp-mediated recombi-

nation, 12 transformants were further grown for 72 h in liquid medium with xylose as a carbon source but lacking any antibiotics. Subsequently, these transformants were transferred onto solid medium with or without nourseothricin but lacking xylose in both cases. We found that 2 out of 12 transformants had lost nourseothricin resistance, and these were designated PcFLIP2 T1 and PcFLIP2 T3 (Fig. 5A). In the following PCR analyses (Fig. 5B), the lack of the *nat1* flipper cassette was confirmed with the primer pairs as indicated. The whole flipper cassette is still present in strains (PcFLIP1) that grow on glucose as a noninducible carbon source. After induction on xylose, the 0.3-kb amplicon, carrying only the recombinant *FRT* sequence, can be detected in the PcFLIP2 transformants. Thus, with the optimized FLP/*FRT* recombination system described here, it is possible to eliminate and to recycle any marker using a one-step approach.
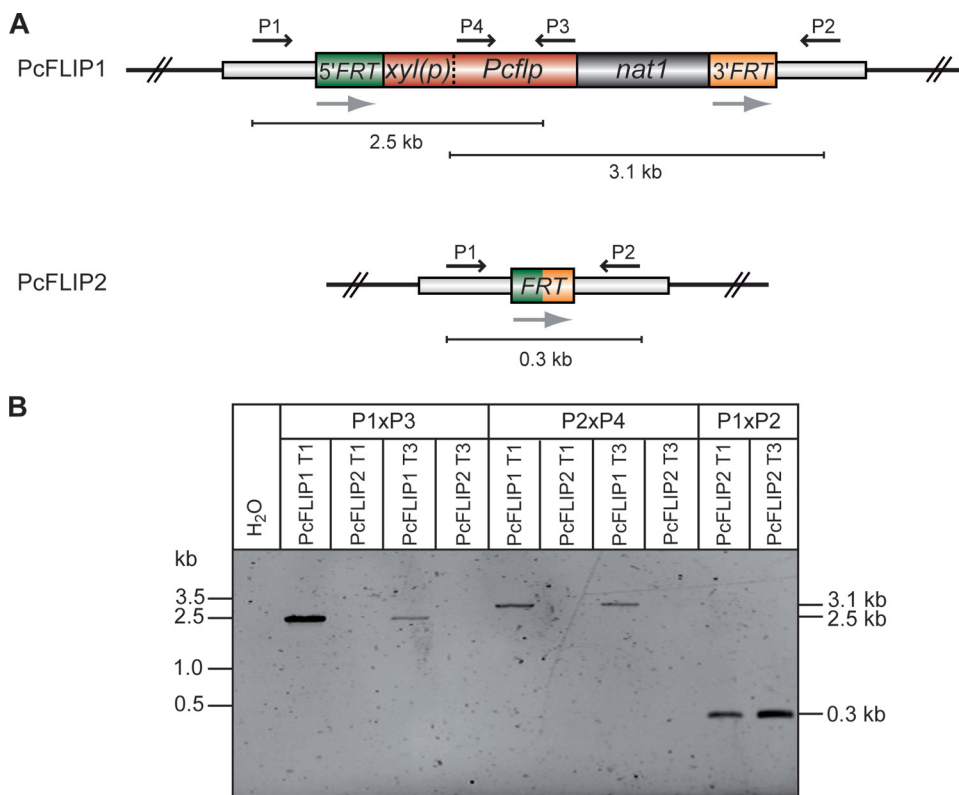
FIG. 5. One-step marker excision. (A) Map of the *nat1* flipper cassette together with the location of primers for PCR analysis before (top) and after (bottom) excision. The location of primers for panel B is given. (B) PCR analysis with DNA from strains as indicated. *xyl(p)*, *xyl* promoter of *P. chrysogenum* P1 × P3, P2 × P4, and P1 × P2 indicate primer pairs used for PCR amplification.

Establishment of the FLP/*FRT* recombination system in *S. macrospora*. To extend the application of the optimized FLP/*FRT* recombination system to a further filamentous fungus, we chose the model ascomycete *S. macrospora* (46), which belongs to the *Sordariomycetes* and is distantly related to *P. chrysogenum*, a member of the *Eurotiomycetes*. For our analysis, we used plasmids which were successfully tested in *P. chrysogenum* as described above. First, the 1.3-kb *FRT-nat1* cassette was ectopically integrated into the wild-type strain to generate primary SmFRT1 transformants. The codon-adapted *Pcflp* recombinase gene was then introduced into three of the primary SmFRT1 transformants using plasmid pGPS2.1-Pcflp conferring hygromycin resistance. Characterization of the 53 resulting secondary SmFRT2P transformants revealed that 26 were unable to grow on selection medium containing nourseothricin; thus, excision of the *FRT-nat1* cassette by the Pcflp recombinase resulted in the loss of the *nat1* gene. To verify this recombination event, a PCR analysis with the primer pair FN_s and FN_a was performed. As shown in Fig. 6, the 1.3-kb *FRT-nat1* cassette was detectable in only the primary SmFRT1 transformants, while all nourseothricin-sensitive secondary SmFRT2P transformants carry only the 0.2-kb amplicon, indicating a successful recombination event in *S. macrospora*. It can be foreseen that the optimized FLP/*FRT* recombination system will be applied to fungi unrelated to the two species investigated in this study.

DISCUSSION

The complete genome sequences of a large number of filamentous fungi are already available, thereby enabling the application of fundamental functional genetic studies. One approach will be the analysis of candidate genes, e.g., involved in the regulation of metabolism and/or morphogenesis. A com-
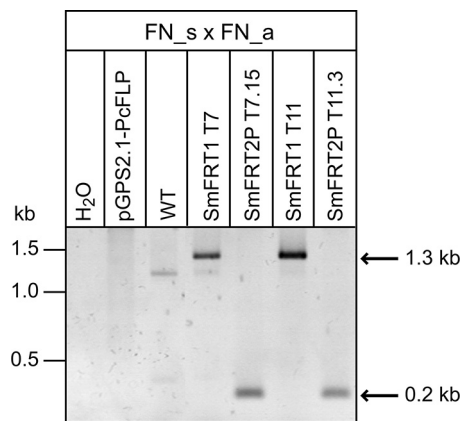

FIG. 6. PCR analysis of *S. macrospora* transformants with primers as indicated. The location of both primers is shown in Fig. 2A. WT, wild type. FN_s × FN_a indicates the primer pair used for PCR amplification.

mon and frequently used strategy in fungal genetics is the disruption of the primary gene structure by insertion or substitution of marker genes using homologous recombination systems (33). Usually, the number of gene disruptions followed by complementation experiments within a single strain is dependent on the available marker genes applicable to a given fungus. One option to generate strains with multiple gene substitutions is sexual recombination; however, this is not possible for most biotechnically relevant fungi since they usually propagate exclusively by asexual means. Note that the FLP/ *FRT* recombination system established here should be applicable to any given filamentous fungus.

Efficient expression of codon-adapted genes in filamentous fungi. Filamentous fungi are often used as producers for homo- and heterologous proteins because of their capability to synthesize and secrete huge amounts of diverse products (8, 27, 42, 59). Heterologous gene expression is especially important for increased production of pharmaceuticals and food additives because often the extraction of these compounds from their natural sources is not cost-effective or production in the corresponding hosts is impossible (4, 67). Unfortunately, however, the yield of most heterologous proteins is generally far lower than that of homologous proteins (8). To solve this problem, different approaches have been developed, including multicopy integration of the coding gene, use of strong regulatory promoters, and fusion with carrier proteins (3, 38). Moreover, codon usage has been identified as an important factor for efficient gene expression, indicating that differences in the codon usage between two species, in general, and between prokaryotes and eukaryotes, in particular, can result in translation inefficiency (20, 27, 36, 59). Because of this, strategies to remove codons from the native gene that are rare in the new host or the introduction of tRNAs for rare codons into the genome of a given organism have been developed (4, 68, 71). Another alternative is the complete synthesis of recombinant genes, an option which has become affordable recently, making the adaption of a complete heterologous gene to the codon usage or codon bias of the organism of choice possible.

Recent examples from fungal systems include the complete reconstruction of the firefly luciferase gene, resulting in a significant increase in gene expression in *Neurospora crassa* (17). Another example from the fungal field is the novel synthesis of the Der f 7 gene encoding a mite allergen. This synthetic gene was fused with the glucoamylase gene for optimal expression in *Aspergillus oryzae*, offering the possibility to produce recombinant allergens for immunotherapy (59). Both studies demonstrate that codon optimization is a powerful strategy to improve the level of gene expression to allow successful protein synthesis in a given host. To establish the FLP/*FRT* recombination system in *P. chrysogenum*, we have optimized the codon usage of the native *flp* recombinase gene from *S. cerevisiae*, an approach which was previously done for optimal expression in the prokaryote *Mycobacterium bovis* (56). This was necessary since expression of the yeast *flp* gene was not sufficient to generate any nourseothricin-sensitive single-spore isolates out of 300 tested spores. A more practical system was obtained with the codon-optimized *Pcflp* version, resulting in expression levels for efficient excision of the *FRT-nat1* cassette in *P. chrysogenum*. Thus, the adapted codon usage most likely allows the translational system to be optimized. Preferred codons usually

correlate with the abundance of cognate tRNAs within a cell, a prerequisite to balance codon with isoacceptor tRNA concentrations. Differences in codon usage were shown previously to be significant obstacles for efficient heterologous gene expression (20). We observed variable frequencies of excision between individual experimental approaches. Different promoters in front of the *Pcflp* gene may result in different expression levels, which are responsible for the variable excision frequency. For example the *xyl* promoter was activated for only 72 h, and longer incubation times might result in higher excision frequencies. Another explanation might be the different host strains, e.g., P2niaD18 versus ΔPcku70, used as recipients in different experiments.

Generation of marker-free transgenic strains. The application of the marker recycling system in the two filamentous fungi *P. chrysogenum* and *S. macrospora* opens up the possibility of wider usage in diverse fungal systems. Such an application will be an attractive option for applied microbiologists when genetically engineered fungal strains are needed in biotechnical production processes. Moreover, as the recombinant strains will be marker free, there should be no problem in fulfilling all essential safety requirements needed to obtain marketing approval. We have previously shown that homologous resistance markers are suitable tools to construct recombinant strains devoid of foreign DNA sequences. Using the homologous β-tubulin gene from the cephalosporin C producer *Acremonium chrysogenum*, we generated a mutated gene version that confers resistance against the fungicide benomyl (44). Using a linear DNA fragment carrying the mutated β-tubulin gene, we were able to generate transgenic strains lacking any heterologous DNA. Similarly, pyrithiamine resistance-conferring riboswitches from fungi can be used as homologous selection markers (30). In this case, the resistance marker resides within the fungal genome and can be used only once. Alternative systems that allow elimination of homologous as well as heterologous marker genes have been developed for microorganisms and plants. For example, cotransformation of two separate DNAs can be conducted; one incorporates a gene of interest, and the other is a self-replicating plasmid carrying the marker.

We have recently found for a filamentous fungus that self-replicating plasmids can be eliminated from a host strain when it is kept on nonselective growth medium (Hoff, unpublished data). In another approach, intragenomic relocation of transgenes via transposable elements was used to eliminate selectable marker genes in filamentous fungi (69). A similar study used a counter-selectable marker flanked by direct repeats to reestablish a functional allele in a transgenic strain (43). The most elegant systems use site-specific recombinases for efficient marker recycling. As demonstrated here and shown by others, marker-free knockout strains can be generated carrying only small sequences of foreign DNA (14–15, 29). Here, we clearly demonstrated how highly applicable this system is by generating a ΔPcku70FRT2 strain devoid of any heterologous gene. Ku70 is an important component of the nonhomologous end-joining (NHEJ) pathway, a mechanism employed by filamentous fungi to repair double-strand breaks. The deletion of the *ku70* gene, a component of the NHEJ pathway, leads to much higher integration frequencies of foreign DNA by homologous recombination, making the construction of knockout

strains a relatively straightforward approach (33). Moreover, the optimized recombination system offers recycling of the integrated resistance marker in a Δ*ku70* background, thus allowing the possibility for fast and efficient production of multiple deletion strains as the availability of suitable marker genes is no longer the limiting factor. All comparable approaches with filamentous fungi have used the Cre/*loxP* recombination system of bacteriophage P1. For example, Krappmann et al. (29) were able to generate a double knockout mutant in *Aspergillus fumigatus* by integrating the Cre/*loxP* system in a two-step approach. A resistance cassette flanked by *loxP* sequences and a self-replicating plasmid carrying the *cre* gene were used to delete the *pabaA* gene encoding the para-aminobenzoic acid (PABA) synthetase, which is involved in virulence. After marker rescue by Cre-mediated recombination, these investigators used the system a second time to remove the *veA* gene encoding a developmental protein.

Application potential of the recombination systems. The FLP/*FRT* recombination system provides multiple options to manipulate the genome of a chosen organism. In addition to the efficient deletion of genes followed by marker recycling as described in this report, integration of genes at defined loci (24), gene tagging at the endogenous locus (49), and transient gene inactivation (60) are useful applications to manipulate organisms genetically.

To establish an efficient and safe recombination system, it is important to control the time point of marker gene excision mediated by the recombinase. To achieve this, different approaches have been developed. In mouse, for example, two independent strains were generated, one containing the marker gene flanked by the recombination sites and the other one harboring the recombinase gene under the control of a tissue- or cell type-specific promoter. After crossing of the two strains, introduction of the recombination system results in site-specific recombination of the marker gene in a specific tissue (28). In addition to this application, an inducible Cre/*loxP* system has been reported for *A. nidulans* that allows consecutive deletions to be introduced (15). In this system, the *cre* gene was placed under the control of the xylose-inducible *xlnA* promoter, which can be repressed on medium containing glucose. Development of an appropriate protocol enables the efficient generation of sequential deletions in this fungus. However, this system uses a two-step strategy since the available components are located on two different DNA constructs. An alternative and efficient one-step strategy was recently applied for *Candida* species. Different versions of so-called flipper cassettes flanked by the *FRT* sequences and containing both the resistance gene and the inducible recombinase gene were generated (13, 40, 50, 57, 60, 66). Using a similar strategy, we generated the *nat1* flipper cassette for *P. chrysogenum*. To prevent a spontaneous loss of the flipper, we used the inducible *xyl* promoter from *P. chrysogenum*, which was shown recently to be a valuable tool for the controlled off/on regulation of gene expression (25). After only 72 h of induction we obtained *P. chrysogenum* strains that had lost the flipper cassette. However, it remains to be investigated whether longer induction times lead to higher recombination efficiencies.

In conclusion, we have demonstrated by a two-step strategy that the established FLP/*FRT* system enables the production of marker-free deletion strains. This provides the opportunity in future experiments to use the *nat1* flipper in a fast and efficient one-step approach for targeted gene excision.

REFERENCES

1. **Abremski, K., and R. Hoess.** 1984. Bacteriophage P1 site-specific recombination. Purification and properties of the Cre recombinase protein. J. Biol. Chem. **259:**1509–1514.
2. **Amin, A., H. Roca, K. Luetke, and P. D. Sadowski.** 1991. Synapsis, strand scission, and strand exchange induced by the FLP recombinase: analysis with half-FRT sites. Mol. Cell. Biol. **11:**4497–4508.
3. **Archer, D. B., I. F. Connerton, and D. A. MacKenzie.** 2008. Filamentous fungi for production of food additives and processing aids. Adv. Biochem. Eng. Biotechnol. **111:**99–147.
4. **Berlec, A., Z. Jevnikar, A. C. Majhenic, I. Rogelj, and B. Strukelj.** 2006. Expression of the sweet-tasting plant protein brazzein in *Escherichia coli* and *Lactococcus lactis*: a path toward sweet lactic acid bacteria. Appl. Microbiol. Biotechnol. **73:**158–165.
5. **Birling, M. C., F. Gofflot, and X. Warot.** 2009. Site-specific recombinases for manipulation of the mouse genome. Methods Mol. Biol. **561:**245–263.
6. **Blakely, G. W., A. O. Davidson, and D. J. Sherratt.** 2000. Sequential strand exchange by XerC and XerD during site-specific recombination at *dif*. J. Biol. Chem. **275:**9930–9936.
7. **Bullock, W. O., J. M. Fernandez, and J. M. Short.** 1987. XL1-Blue—a high-efficiency plasmid transforming *recA Escherichia coli* strain with β-galactosidase selection. Biotechniques **5:**376–378.
8. **Cardoza, R. E., S. Gutierrez, N. Ortega, A. Colina, J. Casqueiro, and J. F. Martin.** 2003. Expression of a synthetic copy of the bovine chymosin gene in *Aspergillus awamori* from constitutive and pH-regulated promoters and secretion using two different pre-pro sequences. Biotechnol. Bioeng. **83:**249–259.
9. **Chen, Y., and P. A. Rice.** 2003. New insight into site-specific recombination from Flp recombinase-DNA structures. Annu. Rev. Biophys. Biomol. Struct. **32:**135–159.
10. **Chiang, C. J., P. T. Chen, and Y. P. Chao.** 2008. Replicon-free and markerless methods for genomic insertion of DNAs in phage attachment sites and controlled expression of chromosomal genes in *Escherichia coli*. Biotechnol. Bioeng. **101:**985–995.
11. **Ding, C., and G. Butler.** 2007. Development of a gene knockout system in *Candida parapsilosis* reveals a conserved role for *BCR1* in biofilm formation. Eukaryot. Cell **6:**1310–1319.
12. **Dreyer, J., H. Eichhorn, E. Friedlin, H. Kürnsteiner, and U. Kück.** 2007. A homologue of the *Aspergillus velvet* gene regulates both cephalosporin C biosynthesis and hyphal fragmentation in *Acremonium chrysogenum*. Appl. Environ. Microbiol. **73:**3412–3422.
13. **Dunkel, N., J. Blass, P. D. Rogers, and J. Morschhäuser.** 2008. Mutations in the multi-drug resistance regulator MRR1, followed by loss of heterozygosity, are the main cause of MDR1 overexpression in fluconazole-resistant *Candida albicans* strains. Mol. Microbiol. **69:**827–840.
14. **Florea, S., K. Andreeva, C. Machado, P. M. Mirabito, and C. L. Schardl.** 2009. Elimination of marker genes from transformed filamentous fungi by unselected transient transfection with a Cre-expressing plasmid. Fungal Genet. Biol. **46:**721–730.
15. **Forment, J. V., D. Ramon, and A. P. MacCabe.** 2006. Consecutive gene deletions in *Aspergillus nidulans*: application of the Cre/*loxP* system. Curr. Genet. **50:**217–224.
16. **Futcher, A. B.** 1986. Copy number amplification of the 2 μm circle plasmid of *Saccharomyces cerevisiae*. J. Theor. Biol. **119:**197–204.
17. **Gooch, V. D., A. Mehra, L. F. Larrondo, J. Fox, M. Touroutoutoudis, J. J. Loros, and J. C. Dunlap.** 2008. Fully codon-optimized *luciferase* uncovers novel temperature characteristics of the *Neurospora* clock. Eukaryot. Cell **7:**28–37.
18. **Grote, A., K. Hiller, M. Scheer, R. Munch, B. Nortemann, D. C. Hempel, and D. Jahn.** 2005. JCat: a novel tool to adapt codon usage of a target gene to its potential expression host. Nucleic Acids Res. **33:**W526–W531.
19. **Groth, A. C., and M. P. Calos.** 2004. Phage integrases: biology and applications. J. Mol. Biol. **335:**667–678.
20. **Gustafsson, C., S. Govindarajan, and J. Minshull.** 2004. Codon bias and heterologous protein expression. Trends Biotechnol. **22:**346–353.

21. **Haas, H., K. Angermayr, I. Zadra, and G. Stoffler.** 1997. Overexpression of *nreB*, a new GATA factor-encoding gene of *Penicillium chrysogenum*, leads to repression of the nitrate assimilatory gene cluster. J. Biol. Chem. **272:**22576–22582.

22. **Hoff, B., J. Kamerewerd, C. Sigl, I. Zadra, and U. Kück.** 2010. Homologous recombination in the antibiotic producer *Penicillium chrysogenum*: strain ΔPcku70 shows up-regulation of genes from the HOG pathway. Appl. Microbiol. Biotechnol. **85:**1081–1094.

23. **Hoff, B., S. Pöggeler, and U. Kück.** 2008. Eighty years after its discovery, Fleming's *Penicillium* strain discloses the secret of its sex. Eukaryot. Cell **7:**465–470.

24. **Huang, L. C., E. A. Wood, and M. M. Cox.** 1997. Convenient and reversible site-specific targeting of exogenous DNA into a bacterial chromosome by use of the FLP recombinase: the FLIRT system. J. Bacteriol. **179:**6076–6083.

25. **Janus, D., B. Hoff, and U. Kück.** 2009. Evidence for Dicer-dependent RNA interference in the industrial penicillin producer *Penicillium chrysogenum*. Microbiology **155:**3946–3956.

26. **Kilby, N. J., M. R. Snaith, and J. A. Murray.** 1993. Site-specific recombinases: tools for genome engineering. Trends Genet. **9:**413–421.

27. **Koda, A., T. Bogaki, T. Minetoki, and M. Hirotsune.** 2005. High expression of a synthetic gene encoding potato alpha-glucan phosphorylase in *Aspergillus niger*. J. Biosci. Bioeng. **100:**531–537.

28. **Kos, C. H.** 2004. Cre/*loxP* system for generating tissue-specific knockout mouse models. Nutr. Rev. **62:**243–246.

29. **Krappmann, S., O. Bayram, and G. H. Braus.** 2005. Deletion and allelic exchange of the *Aspergillus fumigatus veA* locus via a novel recyclable marker module. Eukaryot. Cell **4:**1298–1307.

30. **Kubodera, T., N. Yamashita, and A. Nishimura.** 2000. Pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*: cloning, characterization and application as a dominant selectable marker for transformation. Biosci. Biotechnol. Biochem. **64:**1416–1421.

31. **Kubodera, T., N. Yamashita, and A. Nishimura.** 2002. Transformation of *Aspergillus* sp. and *Trichoderma reesei* using the pyrithiamine resistance gene (*ptrA*) of *Aspergillus oryzae*. Biosci. Biotechnol. Biochem. **66:**404–406.

32. **Kück, U., and B. Hoff.** 2006. Application of the nourseothricin acetyltransferase (*nat1*) as dominant marker for the transformation of filamentous fungi. Fungal Genet. Newsl. **53:**9–11.

33. **Kück, U., and B. Hoff.** 2010. New tools for the genetic manipulation of filamentous fungi. Appl. Microbiol. Biotechnol. **86:**51–62.

34. **Kück, U., S. Pöggeler, M. Nowrousian, N. Nolting, and I. Engh.** 2009. *Sordaria macrospora*, a model system for fungal development, p. 17–39. *In* T. Anke and D. Weber (ed.), The Mycota XV: physiology and genetics. Springer-Verlag, Berlin, Germany.

35. **Landy, A.** 1989. Dynamic, structural, and regulatory aspects of lambda site-specific recombination. Annu. Rev. Biochem. **58:**913–949.

36. **Lithwick, G., and H. Margalit.** 2003. Hierarchy of sequence-dependent features associated with prokaryotic translation. Genome Res. **13:**2665–2673.

37. **Luo, H., and A. P. Kausch.** 2002. Application of FLP/*FRT* site-specific DNA recombination system in plants. Genet. Eng. (N. Y.) **24:**1–16.

38. **Meyer, V.** 2008. Genetic engineering of filamentous fungi-progress, obstacles and future trends. Biotechnol. Adv. **26:**177–185.

39. **Minuth, W., P. Tudzynski, and K. Esser.** 1982. Extrachromosomal genetics of *Cephalosporium acremonium*. 1. Characterization and mapping of mitochondrial-DNA. Curr. Genet. **5:**227–231.

40. **Morschhäuser, J., S. Michel, and P. Staib.** 1999. Sequential gene disruption in *Candida albicans* by FLP-mediated site-specific recombination. Mol. Microbiol. **32:**547–556.

41. **Morschhäuser, J., P. Staib, and G. Köhler.** 2005. Targeted gene deletion in *Candida albicans* wild-type strains by MPAR flipping. Methods Mol. Med. **118:**35–44.

42. **Nelson, G., O. Kozlova-Zwinderman, A. J. Collis, M. R. Knight, J. R. Fincham, C. P. Stanger, A. Renwick, J. G. Hessing, P. J. Punt, C. A. van den Hondel, and N. D. Read.** 2004. Calcium measurement in living filamentous fungi expressing codon-optimized aequorin. Mol. Microbiol. **52:**1437–1450.

43. **Nielsen, J. B., M. L. Nielsen, and U. H. Mortensen.** 2008. Transient disruption of non-homologous end-joining facilitates targeted genome manipulations in the filamentous fungus *Aspergillus nidulans*. Fungal Genet. Biol. **45:**165–170.

44. **Nowak, C., R. Radzio, and U. Kück.** 1995. DNA-mediated transformation of a fungus employing a vector devoid of bacterial DNA sequences. Appl. Microbiol. Biotechnol. **43:**1077–1081.

45. **Nowrousian, M., S. Masloff, S. Pöggeler, and U. Kück.** 1999. Cell differentiation during sexual development of the fungus *Sordaria macrospora* requires ATP citrate lyase activity. Mol. Cell. Biol. **19:**450–460.

46. **Nowrousian, M., J. E. Stajich, M. Chu, I. Engh, E. Espagne, K. Halliday, J. Kamerewerd, F. Kempken, B. Knab, H. C. Kuo, H. D. Osiewacz, S. Poggeler, N. D. Read, S. Seiler, K. M. Smith, D. Zickler, U. Kück, and M. Freitag.** 2010. *De novo* assembly of a 40 Mb eukaryotic genome from short sequence reads: *Sordaria macrospora*, a model organism for fungal morphogenesis. PLoS Genet. **6:**e1000891.

47. **Pel, H. J., J. H. de Winde, D. B. Archer, P. S. Dyer, G. Hofmann, P. J. Schaap, G. Turner, R. P. de Vries, R. Albang, K. Albermann, M. R.** Andersen, J. D. Bendtsen, J. A. Benen, M. van den Berg, S. Breestraat, M. X. Caddick, R. Contreras, M. Cornell, P. M. Coutinho, E. G. Danchin, A. J. Debets, P. Dekker, P. W. van Dijck, A. van Dijk, L. Dijkhuizen, A. J. Driessen, C. d'Enfert, S. Geysens, C. Goosen, G. S. Groot, P. W. de Groot, T. Guillemette, B. Henrissat, M. Herweijer, J. P. van den Hombergh, C. A. van den Hondel, R. T. van der Heijden, R. M. van der Kaaij, F. M. Klis, H. J. Kools, C. P. Kubicek, P. A. van Kuyk, J. Lauber, X. Lu, M. J. van der Maarel, R. Meulenberg, H. Menke, M. A. Mortimer, J. Nielsen, S. G. Oliver, M. Olsthoorn, K. Pal, N. N. van Peij, A. F. Ram, U. Rinas, J. A. Roubos, C. M. Sagt, M. Schmoll, J. Sun, D. Ussery, J. Varga, W. Vervecken, P. J. van de Vondervoort, H. Wedler, H. A. Wösten, A. P. Zeng, A. J. van Ooyen, J. Visser, **and H. Stam.** 2007. Genome sequencing and analysis of the versatile cell factory *Aspergillus niger* CBS 513.88. Nat. Biotechnol. **25:**221–231.

48. **Pöggeler, S., M. Nowrousian, S. Jacobsen, and U. Kück.** 1997. An efficient procedure to isolate fungal genes from an indexed cosmid library. J. Microbiol. Methods **29:**49–61.

49. **Prein, B., K. Natter, and S. D. Kohlwein.** 2000. A novel strategy for constructing N-terminal chromosomal fusions to green fluorescent protein in the yeast *Saccharomyces cerevisiae*. FEBS Lett. **485:**29–34.

50. **Reuss, O., A. Vik, R. Kolter, and J. Morschhäuser.** 2004. The SAT1 flipper, an optimized tool for gene disruption in *Candida albicans*. Gene **341:**119–127.

51. **Sadowski, P.** 1986. Site-specific recombinases: changing partners and doing the twist. J. Bacteriol. **165:**341–347.

52. **Sambrook, J., and D. W. Russell.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

53. **Schweizer, H. P.** 2003. Applications of the *Saccharomyces cerevisiae* Flp-*FRT* system in bacterial genetics. J. Mol. Microbiol. Biotechnol. **5:**67–77.

54. **Senecoff, J. F., R. C. Bruckner, and M. M. Cox.** 1985. The FLP recombinase of the yeast 2-μm plasmid: characterization of its recombination site. Proc. Natl. Acad. Sci. U. S. A. **82:**7270–7274.

55. **Sharan, S. K., L. C. Thomason, S. G. Kuznetsov, and D. L. Court.** 2009. Recombineering: a homologous recombination-based method of genetic engineering. Nat. Protoc. **4:**206–223.

56. **Song, H., and M. Niederweis.** 2007. Functional expression of the Flp recombinase in *Mycobacterium bovis* BCG. Gene **399:**112–119.

57. **Staib, P., M. Kretschmar, T. Nichterlein, H. Hof, and J. Morschhäuser.** 2000. Differential activation of a *Candida albicans* virulence gene family during infection. Proc. Natl. Acad. Sci. U. S. A. **97:**6102–6107.

58. **Theodosiou, N. A., and T. Xu.** 1998. Use of FLP/*FRT* system to study *Drosophila* development. Methods **14:**355–365.

59. **Tokuoka, M., M. Tanaka, K. Ono, S. Takagi, T. Shintani, and K. Gomi.** 2008. Codon optimization increases steady-state mRNA levels in *Aspergillus oryzae* heterologous gene expression. Appl. Environ. Microbiol. **74:**6538–6546.

60. **Ueno, K., J. Uno, H. Nakayama, K. Sasamoto, Y. Mikami, and H. Chibana.** 2007. Development of a highly efficient gene targeting system induced by transient repression of *YKU80* expression in *Candida glabrata*. Eukaryot. Cell **6:**1239–1247.

61. **van den Berg, M. A., R. Albang, K. Albermann, J. H. Badger, J. M. Daran, A. J. Driessen, C. Garcia-Estrada, N. D. Fedorova, D. M. Harris, W. H. Heijne, V. Joardar, J. A. Kiel, A. Kovalchuk, J. F. Martin, W. C. Nierman, J. G. Nijland, J. T. Pronk, J. A. Roubos, I. J. van der Klei, N. N. van Peij, M. Veenhuis, H. von Döhren, C. Wagner, J. Wortman, and R. A. Bovenberg.** 2008. Genome sequencing and analysis of the filamentous fungus *Penicillium chrysogenum*. Nat. Biotechnol. **26:**1161–1168.

62. **Van Duyne, G. D.** 2001. A structural view of cre-*loxp* site-specific recombination. Annu. Rev. Biophys. Biomol. Struct. **30:**87–104.

63. **Veit, B. E., and W. L. Fangman.** 1988. Copy number and partition of the *Saccharomyces cerevisiae* 2μm plasmid controlled by transcription regulators. Mol. Cell. Biol. **8:**4949–4957.

64. **Vetter, D., B. J. Andrews, L. Roberts-Beatty, and P. D. Sadowski.** 1983. Site-specific recombination of yeast 2-μm DNA *in vitro*. Proc. Natl. Acad. Sci. U. S. A. **80:**7284–7288.

65. **Voziyanov, Y., S. Pathania, and M. Jayaram.** 1999. A general model for site-specific recombination by the integrase family recombinases. Nucleic Acids Res. **27:**930–941.

66. **Wirsching, S., S. Michel, and J. Morschhäuser.** 2000. Targeted gene disruption in *Candida albicans* wild-type strains: the role of the MDR1 gene in fluconazole resistance of clinical *Candida albicans* isolates. Mol. Microbiol. **36:**856–865.

67. **Yang, B., Z. Guo, Y. Huang, and S. Zhu.** 2004. Codon optimization of MTS1 and its expression in *Escherichia coli*. Protein Expr. Purif. **36:**307–311.

68. **Yang, Y., M. Malten, A. Grote, D. Jahn, and W. D. Deckwer.** 2007. Codon optimized *Thermobifida fusca* hydrolase secreted by *Bacillus megaterium*. Biotechnol. Bioeng. **96:**780–794.

69. **Yoder, J. I., and A. P. Goldsbrough.** 1994. Transformation systems for generating marker-free transgenic plants. Nat. Biotechnol. **12:**263–267.

70. **Zadra, I., B. Abt, W. Parson, and H. Haas.** 2000. *xylP* promoter-based expression system and its use for antisense downregulation of the *Penicillium chrysogenum* nitrogen regulator NRE. Appl. Environ. Microbiol. **66:**4810–4816.

71. **Zhou, Z., P. Schnake, L. Xiao, and A. A. Lal.** 2004. Enhanced expression of a recombinant malaria candidate vaccine in *Escherichia coli* by codon optimization. Protein Expr. Purif. **34:**87–94.