

# Deletion and Allelic Exchange of the *Aspergillus fumigatus* *veA* Locus via a Novel Recyclable Marker Module

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**Detailed evaluation of gene functions in an asexual fungus requires advanced methods of molecular biology. For the generation of targeted gene deletions in the opportunistic pathogen *Aspergillus fumigatus* we designed a novel blaster module allowing dominant selection of transformants due to resistance to phleomycin as well as dominant (counter)selection of a Cre recombinase-mediated marker excision event. For validation purposes we have deleted the *A. fumigatus* *pabaA* gene in a wild-type isolate by making use of this cassette. The resulting *pabaA::loxP* strain served as the recipient for subsequent targeting of the *velvet* locus. Homologous reconstitution of the deleted gene was performed by an allele whose expression is driven in a nitrogen source-dependent manner, as validated by Northern analyses. Overexpression of the *veA* locus in *A. fumigatus* does not result in any obvious phenotype, whereas the sporulation capacities of the *veA* null mutant are reduced on nitrate-containing medium, a phenotype that is completely restored in the reconstituted strain.**

With the advent of a steadily increasing number of microbial genome sequences, molecular tools to assign gene functions are a necessity in future basic research. Within the plethora of genome sequencing projects, fungal genomes have always been of special relevance: the ascomycete *Saccharomyces cerevisiae* was the first eukaryote whose genomic content was determined (23), lately complemented by efforts to resolve the complete genetic information from its filamentous ancestor *Ashbya gossypii* (15). The genome sequence of the model organism *Neurospora crassa* has been annotated recently (4, 21), and the genomes of three aspergilli, *Aspergillus (Emericella) nidulans*, *A. fumigatus*, and *A. oryzae*, are currently under comparative investigation.

One of the major obstacles in evaluation of any eukaryotic genome sequence lies in the identification of loci encoding potential gene products. Comparative BLAST studies may identify conserved loci that are likely to be expressed. Comparison of stretches of genomic sequences with entries in expressed sequence tag (EST) databases or cDNA sequences serves to identify expressed loci together with their exon/intron architecture (2). Gene functions might be assessed via identification of well-characterized orthologues from comprehensive databases; nevertheless, the function of a large fraction of the annotated genes from any newly determined genome sequence remains putative, as exemplified by the 41% of predicted *Neurospora crassa* proteins lacking significant similarity to gene products from the public databases (21).

A first glance on gene functions is achieved by the generation of null mutations in the organism under investigation, provided that adequate screening for phenotypes of interest is available. An established molecular biology is a vital prerequisite for controlled manipulation of any organism on the

genomic level, and targeted gene manipulation by means of precise gene replacement has become the method of choice in addressing gene functions and characteristics. Genetic manipulation of deuteromycetous fungi is restricted due to the absence of any mode of sexual propagation, which would be accompanied by meiotic recombination. Therefore, genetic markers have to be introduced by transformation or by exploiting any parasexual cycle (20, 59). Furthermore, when addressing factors that might contribute to the virulence of a pathogen, dominance of the genetic markers is desirable to exclude effects that might be solely based on imperfect complementation of the genetic lesion (6).

Saprophytes of the genus *Aspergillus* have become one of the most relevant opportunistic fungal pathogens in present times (38). Based on advances in immunosuppressive medical treatment, the incidence of aspergillosis has increased steadily over the past decade (34), and the vast majority of them are caused by *A. fumigatus* spores. The pathogenicity of *A. fumigatus* is regarded as multifactorial, with a variety of cellular attributes contributing to the virulence potential of this asexual fungus (39). *A. fumigatus* has advanced to a practical level with standard procedures such as transformation, gene disruption and deletion, or integration of reporter fusions being well established (5). In virulence studies, the replacement of any gene under scrutiny by a dominant marker followed by homologous reconstitution with a silently mutated allele is useful to determine its potential as a virulence factor following the proposed molecular postulates of Koch (17, 18). Although the molecular techniques of genetic marker rescue and precise allelic replacement have been evaluated for *Aspergillus* (8, 12, 13, 35), a dominant system of selection and counterselection facilitating this task has not been described to date.

The annotation of the *A. fumigatus* genome sequence has uncovered gene loci formerly characterized in the model representative *A. nidulans* (46). Of special interest are genes that contribute to the sexual propagation mode or fruit body formation of the latter species (7), as their role in the life cycle of

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the asexual fungus *A. fumigatus* remains tentative. One prominent example is represented by the *velvet* locus (30). The nature of the *veA* gene determines the developmental capacities of *A. nidulans*. The Glasgow wild-type strain commonly inherits the wild-type allele of this gene, whereas most laboratory strains express a truncated version encoded by the *veA1* allele. In *A. nidulans* the balance between asexual conidiphore formation and sexual fruit body differentiation is influenced by the nature of the *velvet*-encoded gene product. A wild-type isolate usually forms conidia on a growth substrate under illumination, and cleistothecium formation is preferred in the dark under conditions of restricted air exchange. For *veA1* strains this dependency on distinct environmental conditions is partly diminished, as these strains conidiate profoundly in the absence of light but produce fruiting bodies in a delayed and reduced fashion (11). In its overall appearance, these *veA1* mutant strains do not display the velvety appearance of older *A. nidulans* colonies, and, furthermore, they are blind for red light, which promotes conidiation in a wild-type background (43). Full deletion of the *veA* locus results in a completely acleistothecial phenotype accompanied by the secretion of a dark pigment, whereas overexpression of the *veA* gene product induces the formation of sexual structures under inappropriate conditions (32). Based on these and other findings, the VeA protein was designated a positive-acting factor of *A. nidulans*, promoting the sexual differentiation of this homothallic species. Recent studies have demonstrated a further role of the *veA* gene product in the regulation of secondary metabolism (10, 31), including the control of genes which directly or indirectly participate in carbohydrate metabolism, such as the mannoprotein-encoding gene *mnpA* (27) and fructosyl amine induction of the *faoA* gene, which encodes a fructosyl amino acid oxidase activity (28).

Here we address the role of the *A. fumigatus*-encoded *velvet* gene in the lifestyle of this obligate asexual fungus. By designing a suitable fungal marker module for dominant selection as well as counterselection (41) we aimed at the precise exchange of the endogenous *A. fumigatus veA* locus for a conditional allele by a two-step procedure of gene deletion and marker replacement. For safety reasons these studies were carried out by manipulating a strain of *A. fumigatus* that carries a complete deletion of the *pabaA* locus (8, 54) from which the reusable marker module had been excised by Cre-mediated recombination (55). Inspection of the corresponding *A. fumigatus* strains revealed that overexpression of the *veA* gene does not result in any obvious developmental phenotype, whereas VeA is required for full sporulation capacity in the presence of nitrate as the sole nitrogen source.

#### MATERIALS AND METHODS

**Strains, media, and growth conditions.** The fungal strains used throughout this study are listed in Table 1. The clinical isolate D141 (50) served as the wild-type progenitor for all *A. fumigatus* strains constructed. *Escherichia coli* strains DH5 $\alpha$  (61) and SURE (Stratagene) were employed for preparation of plasmid DNA and were propagated in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl) in the presence of ampicillin at 100  $\mu\text{g ml}^{-1}$ . For recombinogenic engineering (recombineering) purposes (12), the bacterial strain KS272 carrying the pKOBEG plasmid was grown in low-salt LB medium with 0.5% NaCl in the presence of 25  $\mu\text{g ml}^{-1}$  chloramphenicol. Minimal medium (0.52 g liter $^{-1}$  KCl, 0.52 g liter $^{-1}$  MgSO $_4$ , 1.52 g liter $^{-1}$  KH $_2$ PO $_4$ , 0.1% trace element solution [29], pH 6.5) was used for growth of fungal strains, supplemented with appropriate

TABLE 1. Strains used in this study

Strain	Genotype	Reference
D141	<i>A. fumigatus</i> wild type, clinical isolate	50
AfS11	<i>pabaA::loxP-phleo/tk</i>	This study
AfS12	<i>pabaA::loxP</i>	This study
AfS13	<i>veA::loxP-phleo/tk</i>	This study
AfS15	<i>pabaA::loxP veA::loxP-phleo/tk</i>	This study
AfS25	<i>pabaA::loxP<sup>r</sup> niaA::veA</i>	This study

amounts of 4-aminobenzoic acid (PABA, 1  $\mu\text{g ml}^{-1}$ ), phleomycin (30  $\mu\text{g ml}^{-1}$ ), 5-fluoro-2'-deoxyuridine (FUDR, 100  $\mu\text{M}$ ), or pyriithiamine (0.1  $\mu\text{g ml}^{-1}$ ); 1% D-glucose was used as the source of carbon with 10 mM nitrogen source such as ammonium, supplemented as tartrate salt, or sodium nitrate.

**Transformation procedures.** Protocols for *E. coli* were either for calcium- and manganese-treated cells (26) or for electroporation (56) with a Bio-Rad GenePulser at 2.5 kV in 0.2-cm cuvettes. *A. fumigatus* was transformed by polyethylene glycol-mediated fusion of protoplasts essentially as described (49).

**Manipulation of nucleic acids.** Standard protocols of recombinant DNA technology were carried out (53). Recombination of DNA in *E. coli* employing the Redaby system was executed following the procedure of Chaveroche et al. (12). *Taq* and *Pfu* polymerases were generally used in PCRs (52) and essential cloning steps were confirmed by sequencing on an ABI Prism 310 capillary sequencer. Fungal genomic DNAs were prepared from ground mycelia (33), and Southern analyses were carried out as described (57). Samples of total RNA were isolated using the TRIzol reagent of Invitrogen followed by Northern hybridization according to the protocols cited by Brown and Mackey (9). Random-primed labeling of hybridization probes was carried out with the Stratagene Prime-It II kit in the presence of [ $\alpha$ - $^{32}\text{P}$ ]dATP (19). To generate autoradiographs, the washed membranes were exposed to Kodak X-omat films. Sequence analyses were carried out using the Lasergene Biocomputing software package from DNASTar and alignments were created by the Lipman-Pearson method (40).

**Plasmid constructions.** The plasmids used and constructed during the course of this study are listed in Table 2, accompanied by the oligonucleotide sequences recorded in Table 3. Plasmid pME2889 was constructed by inserting an XhoI/NotI fragment from pME2256 in pBluescript II KS (Stratagene). The phleomycin blaster cassette pME2891 was constructed in five steps: an 0.4-kb amplicon generated by PCR on pAN8-1 (49, GenBank accession number Z32751) with primer pair Sv95 (positions 2297 to 2320)/Sv85 (positions 2672 to 2655, plus additional nucleotide to create EcoRV half site) was inserted into the EcoRV site of cloning phagemid pBluescript II KS, followed by insertion of a 1.1-kb fragment amplified from a plasmid containing the coding sequence of herpes simplex virus type 1 (HSV1) thymidine kinase (42) with oligonucleotides Sv86 (positions +4 to +22 of coding sequence) and Sv87 (positions +1131 to +1113). From the resulting plasmid a 1.5-kb NcoI/XbaI fragment was released to replace a 1.1-kb NcoI/XbaI fragment in pAN8-1.

Next, the *trpC* terminator sequence was inserted into this plasmid as a 0.7-kb BamHI/XbaI fragment isolated from pAN7-1 (49). From this construct the resulting 4.4-kb BglII/XbaI *gpdA::ble/tk::trpC* cassette was transferred to a derivative of pUG6 (25) from which the *kanMX* module had been removed after BglII/XhoI digestion, followed by Klenow treatment and religation.

The Cre recombinase expression plasmid pME2892 was assembled as follows. The coding sequence of the *cre* gene was amplified from pSH47 (25) (accession number AF298782) with primer pair OLSK58 (EcoRI site, positions 3673 to 3655)/OLSK59 (BglII, positions 2642 to 2660) and inserted into the EcoRV site of pME2890, which carries the *A. nidulans niaA/niaD* intergenic region as a 1.2-kb KpnI fragment (45) and the *niaD* 3' region (accession number M58291) as an XbaI/SpeI amplicon from genomic DNA via Sv88 (positions 3303 to 3325) and Sv89 (positions 3588 to 3565). The resulting *niaD::cre* module was amplified by PCR and inserted into the SmaI site of the autonomously replicating *Aspergillus* plasmid pPTRII from Takara. Both constructs, pME2891 and pME2892, will be made available at the Fungal Genetics Stock Center (<http://www.fgsc.net>).

Plasmids pME2893 and pME2895 were isolated from partial sublibraries created from genomic DNA of strain D141 that had been fragmented by the restriction endonucleases BamHI and SacII or BglII and NruI, respectively. In pME2894 the *pabaA* coding sequence was exchanged for the *loxP-phleo/tk* blaster module by recombineering linearized pME2893 with the PCR product of primers Sv160 and Sv161 on template pME2891 (12, 37). For construction of pME2896, the 4.5-kb HpaI/HindIII fragment of pME2891 was ligated to the Csp45I/HindIII backbone of pME2895 to yield the *veA::loxP-phleo/tk* deletion cassette. The

TABLE 2. Plasmid constructs employed in this study

Plasmid	Description	Reference
pBluescript II KS	General cloning plasmid ( <i>bla</i> , multiple cloning site)	Stratagene
pUG6	<i>kanMX</i> marker module ( <i>bla loxP-TEF2::kan<sup>r</sup>-loxP</i> )	25
pSH47	Yeast Cre expression plasmid ( <i><sup>p</sup>GAL1::cre URA3 ARS/CEN</i> )	25
pAN7-1	<i>Aspergillus</i> marker cassette conferring resistance to hygromycin [ <i><sup>p</sup>gpdA::hph::trpC'</i> ]	49
pAN8-1	<i>Aspergillus</i> marker cassette conferring resistance to phleomycin [ <i><sup>p</sup>gpdA::ble::trpC'</i> ]	49
pPTRII	Autonomously replicating <i>Aspergillus</i> plasmid ( <i>ptrA AMA1 bla</i> )	Takara
pME2256	Yeast plasmid pRS316 ( <i>URA3, ARS/CEN</i> ) with BglII site in polylinker	Collection of AG Braus
pME2889	pBluescript II KS derivative carrying BglII site in polylinker	This study
pME2890	<i>A. nidulans</i> <i><sup>p</sup>niaD-niaD'</i> expression module in pBluescript II KS	This study
pME2891	<i>loxP-phleo/tk</i> blaster ( <i>loxP-<sup>p</sup>gpdA::ble/HSV1 tk::trpC'-loxP</i> )	This study
pME2892	Cre expression module in pPTRII ( <i>A. nidulans niaD::cre ptrA AMA1</i> )	This study
pME2893	<i>pabaA</i> genomic locus as 9.0-kb BamHI/SacII fragment in pBluescript II KS	This study
pME2894	<i>pabaA</i> deletion cassette ( <i>pabaA::loxP-phleo/tk</i> )	This study
pME2895	<i>veA</i> genomic locus as 6.7-kb BglII/NruI fragment in pME2889 (BglII/SmaI)	This study
pME2896	<i>veA</i> deletion cassette ( <i>veA::loxP-phleo/tk</i> )	This study
pME2897	<i>veA</i> reconstitution allele for promoter insertion ( <i><sup>p</sup>niaA::veA</i> )	This study

pME2897 reconstitution allele to exchange the endogenous *veA* promoter sequence for the *A. fumigatus niaA* 5' region was generated by insertion of a 1.2-kb PCR product amplified with primers Sv233 (BstBI, positions -1222 to -1194 relative to the *niaA* coding sequence) and Sv234 (BstBI, positions -10 to -30) from the genomic DNA of strain D141 into the unique BstBI site of plasmid pME2895.

**Nucleotide sequence accession numbers.** pME2891 and pME2892 were submitted to GenBank under accession no. DQ023271 and DQ023272.

## RESULTS

**Novel blaster module facilitates subsequent gene deletions and reconstitution in *Aspergillus fumigatus*.** The molecular biology of the pathogen *Aspergillus fumigatus* is hampered by the lack of a sexual cycle. Introduction of precise deletions often calls for the reconstitution of the deleted locus in later steps, which is made difficult by the low frequency of homologous recombination in this filamentous fungus. Furthermore, the limited number of marker genes suitable for gene inactivation by gene replacement restricts the number of genetic lesions that can be introduced in a particular recipient strain. We therefore sought an approach to circumvent these general issues and to develop a generally applicable system suitable for targeted gene inactivation accompanied by the possibility of homologous reconstitution (Fig. 1).

In general, this task requires a marker suitable for dominant

negative selection, and based on earlier studies in filamentous fungi, the suicide gene constituted by the thymidine kinase gene of herpes simplex virus type 1 seemed highly appropriate (24, 48, 51). The enzymatic activities encoded by such genes catalyze the phosphorylation of nucleoside analogues, which in turn interfere with the nucleotide metabolism of the host cell. Conclusively, the coding sequence of the HSV1 thymine kinase gene was combined with the *ble* gene sequence as a translational fusion to yield a chimeric dominant marker module allowing positive selection against the antibiotic phleomycin as well as negative selection against the antimetabolite 5-fluoro-2'-deoxyuridine (FUDR). To allow constitutive expression in the fungal host, this sequence stretch was positioned between the *gpdA* promoter sequence and *trpC* termination region of *A. nidulans*, as validated in the well-established expression module of pAN8-1 (49).

In order to expand the practicability of the novel marker we made marker rescue possible by the insertion of inverted repeats upstream and downstream of the genetic marker. These *loxP* sites serve as acceptor sites for the Cre recombinase and mediate excision of sequences sandwiched between them (55). To achieve controlled expression of the *loxP*-specific recombinase, an expression module placing the encoding *cre* gene between the *A. nidulans niaD* 5' and 3' regulatory sequences

TABLE 3. Oligonucleotides used in this study

Designation	Sequence
Sv85.....	5'-ATC CTG CTC CTC GGC CAC G-3'
Sv86.....	5'-GCT TCG TAC CCC TGC CAT-3'
Sv87.....	5'-TCA GTT AGC CTC CCC CAT C-3'
Sv88.....	5'-TTC ATT ACT TCT AGA CGG GTT CG-3'
Sv89.....	5'-AAG AGG TAT TAC TAG TCT ACA GTG-3'
Sv95.....	5'-TCA CCA TGG CCA AGT TGA CCA GTG-3'
Sv160.....	5'-TCA CTC CCC TTT TGG TGT TCT CTG AGT ACC GTC GGA GCT TGC GGG CCA CAG CAA TTA GGC CAC TAG TGG ATC TG-3'
Sv161.....	5'-TGA AGT ATT CAC CTC GAC GTC CAA AAC ATC ATC CAC AAG AAA GAA GAG ATG CTT GCA GCT GAA GCT TCG TAC GC-3'
Sv233.....	5'-TAC ATT CGA AGA GTA CTA CAG CTG ATA ATG TCG GTA TAG-3'
Sv234.....	5'-ATC GTT CGA AGT CTC AAC GCC AGT ATT GAC C-3'
OLSK58.....	5'-TAT AGA ATT CAT GTC CAA TTT ACT GAC CG-3'
OLSK59.....	5'-ATA TAG ATC TCT AAT CGC CAT CTT CCA GC-3'

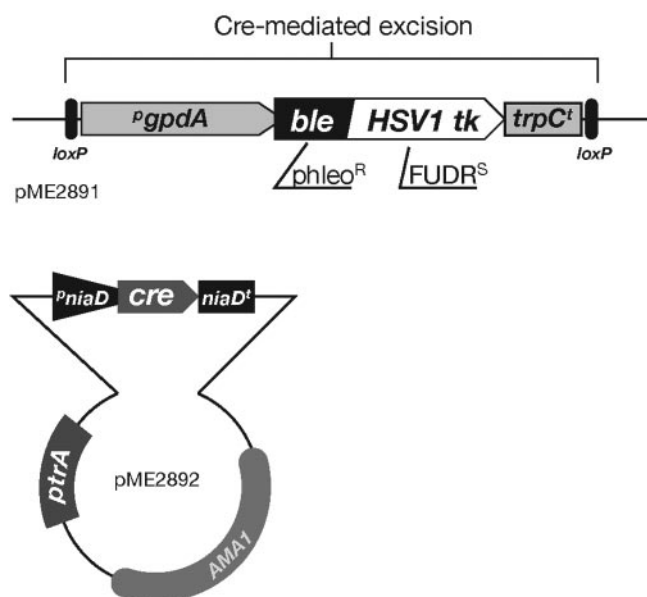


FIG. 1. Plasmid constructs apt for *A. fumigatus* gene deletions and homologous reconstitution. Schematically depicted are the elements of plasmids pME2891 (top) and pME2892 (bottom). The marker module is a derivative of pAN8-1 and includes a translational fusion of the *ble* gene with the thymidine kinase-encoding sequence from herpes simplex virus type 1 (*HSV1 tk*). This chimeric sequence confers resistance to the antibiotic phleomycin ( $phleo^R$ ) as well as sensitivity to nucleoside analogues such as 5-fluoro-2'-deoxyuridine ( $FUDR^S$ ). To accomplish excision by site-specific recombination, *loxP* acceptor sites were added on either edge of the cassette. Transient expression of the Cre recombinase in a nitrogen source-dependent manner can be achieved from the expression module integrated into the autonomously replicating vector pME2892, which carries the *ptrA* marker allele for plasmid maintenance in the presence of pyrithiamine.

was constructed. This *niaD::cre* component was cloned into plasmid pPTRII, suitable for autonomous replication in *Aspergillus* species (36). Both plasmids, pME2891 and pME2892, constitute a novel system for dominant marker selection in a positive as well as negative manner, allowing us to employ the marker in a repeated fashion for subsequent targeting of multiple loci in *Aspergillus fumigatus* in addition to homologous reconstitution of deletion mutant strains (for a detailed protocol outline, see our website at <http://wwwuser.gwdg.de/~molmibio/organigr.htm>).

**Generation of an *A. fumigatus pabaA::loxP* strain.** In order to validate our marker system we targeted the *pabaA* locus of the wild-type clinical isolate D141. This locus encodes the *para*-aminobenzoic acid (PABA) synthetase, which converts chorismate, the last common intermediate of aromatic amino acid biosynthesis, into the folate precursor PABA. PABA-requiring *Aspergillus* mutant strains have been shown in earlier studies to be dramatically impaired in their virulence characteristics, and the *pabaA* gene product was regarded as an attractive drug target to counteract microbial pathogens (8).

The complete locus as deduced from the genome sequence was cloned in plasmid pME2895 from a genomic D141 sublibrary and served as the template in a recombinering approach, which is genetic engineering based on homologous recombination in an *E. coli* host strain expressing phage-de-

rived proteins (44). For that purpose, the *loxP-phleo/tk* module of pME2891 was amplified with oligonucleotides that contained short homology arms of 50 nucleotides complementary to the 5' and 3' untranslated regions of the *pabaA* locus. Cotransformation of linearized plasmid pME2895 with the amplicon into the arabinose-induced *E. coli* host strain KS272/pKOBEG (12) yielded the recombined construct pME2894, in which the *pabaA* coding sequence had been replaced completely by the marker component. This replacement cassette with approximately 4.0-kb 5' and 2.5-kb 3' homologous flanking regions was introduced into strain D141, and primary transformants were selected on phleomycin-containing medium supplemented with PABA.

Detailed inspection of selected candidates after colony purification revealed two isolates that displayed the predicted phenotype: these strains only grow in the presence of the supplement PABA, exhibit resistance to the antibiotic phleomycin, and, furthermore, are impaired in growth on medium containing the nucleoside analogue FUDR (Fig. 2C). From Southern analysis, homologous replacement of the *pabaA* locus by the deletion marker cassette could be confirmed in both transformants (not shown), and one of them was chosen as representative Afs11 for further studies. Using Afs11 as the transformation recipient, the autonomously replicating plasmid pME2892 was put into action, and transformants were allowed to grow in the presence of pyrithiamine. To induce *pniaD*-driven expression of the *cre* gene, several of them were propagated on medium containing nitrate as the sole source of nitrogen, and clonal isolates from this lineage were screened for acquired FUDR resistance due to pop-out of the marker cassette. Out of 20 segregates, five displayed impaired growth on FUDR-containing medium, and all of these isolates were additionally scored as sensitive to phleomycin.

When plating conidia of Afs11 at various densities on FUDR-containing medium, no colonies could be grown (not shown), indicating the absence of spontaneous recombination between the flanking *loxP* sites. Furthermore, the *pabaA::loxP* lesion from two  $FUDR^S$  isolates after Cre expression was amplified from the genome and sequenced to validate precise recombination between the *loxP* sites. Identical sequences from both isolates demonstrated the presence of one *loxP* site flanked by 5' and 3' *pabaA* sequences, as expected (Fig. 2A). To check for loss of the expression plasmid, conidia from these two isolates were streaked out on supplemented medium without the selective agent, and single colonies from these plates were scored for resistance to pyrithiamine. All of these descendants were sensitive to pyrithiamine, indicating a high rate of plasmid loss when selective pressure is relieved. As a representative, one isolate was chosen, and this strain, Afs12, clearly exhibits the phenotype expected, which is proper growth in the presence of FUDR as well as sensitivity to phleomycin, accompanied by PABA auxotrophy (Fig. 2C). Correspondingly, the correct genotypes of both strains, Afs11 and Afs12, could be confirmed in Southern hybridization experiments (Fig. 2B).

In summary, we were able to replace a target gene of *A. fumigatus* by the novel dominant marker module and successfully rescued the genetic marker after precise excision mediated by Cre/*loxP* recombination. The resulting *pabaA::loxP* strain can serve as the recipient in any subsequent gene targeting procedure.

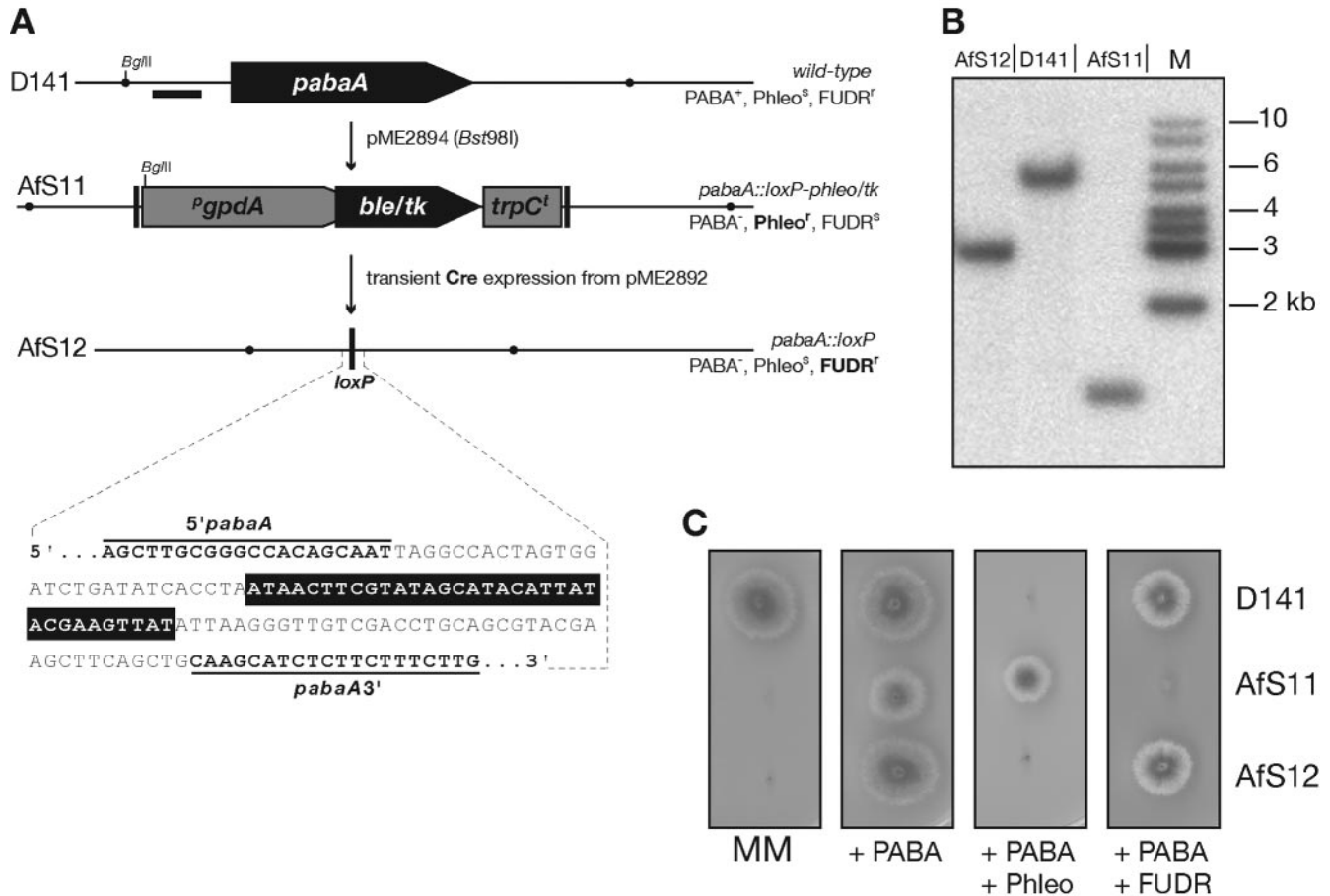


FIG. 2. Deletion of the *A. fumigatus pabaA* locus followed by marker rescue. (A) Schematic representation of the *pabaA* gene as well as the deleted locus prior to and after excision of the *loxP-phleo/tk* marker module. Complete deletion of the *pabaA* coding sequence was achieved by transformation of the wild-type isolate D141 with a replacement cassette from pME2894. Recombination between the flanking *loxP* sites (vertical bars) and rescue of the genetic marker was carried out by transient expression of the Cre recombinase after transformation with pME2892. *Bgl*II restriction sites are indicated by dots; the black horizontal bar indicates the position of the probe used in hybridization experiments. The strains' phenotypes corresponding to the resulting genotypes are indicated. The sequence extract from the genomic *pabaA::loxP* lesion as determined by sequence analyses is given below with *pabaA*-specific 5' and 3' stretches in bold and the *loxP* inverted repeat boxed in black. (B) Southern analysis of D141, AfS11, and AfS12. Equivalent genomic DNA was subjected to *Bgl*III digestion and hybridization signals were detected employing a 5'-specific probe as specified. Fragment sizes of the DNA standard (M) are indicated. (C) Growth phenotypes of the resulting strains D141, AfS11, and AfS12. Conidia were plated on minimal medium (MM) supplemented with 4-aminobenzoic acid (PABA), phleomycin (Phleo), or 5-fluoro-2'-deoxyuridine (FUDR) and grown at 37°C.

***veA* locus is conserved among aspergilli.** To exploit the characteristics of the *loxP-phleo/tk* marker module further we aimed at the deletion of an additional *A. fumigatus* gene in the *pabaA::loxP* background of strain AfS12. As gene of interest the *veA* locus was chosen, based on its significance for fruiting body formation in the homothallic relative *A. nidulans* (43). By inspection of the genome sequence of the *A. fumigatus* isolate Af293, a gene locus could be identified that carries a coding sequence with significant similarity to the *veA* gene of *A. nidulans*. As deduced from the BLAST results that were obtained by submission of this sequence stretch, this genetic locus holds the capacity to encode a polypeptide of 570 amino acids, which is encoded by two exons that are separated by one intronic sequence of 79 nucleotides (Fig. 3A).

In accordance with these in silico assumptions, a complete cDNA could be amplified by reverse transcription-PCR that confirmed the absence of this intron in the processed *veA*

transcript of *A. fumigatus*. The *veA* gene product shows a high degree of conservation with its *A. nidulans* counterpart (Fig. 3B). In the alignment based on the Lipman-Pearson algorithm, the similarity index was calculated to be 53.3%. Only two stretches, one in each deduced amino acid sequence, interfere with proper alignment, which may hint at different molecular characteristics of the proteins. Nevertheless, when transformed into an *A. nidulans* recipient strain deleted of its *veA* locus, the *A. fumigatus* gene restored the phenotypes originating from the genetic lesion (not shown): these reconstituted isolates did not form cleistothecia but did not excrete a dark brown pigment, which is highly characteristic of *A. nidulans* mutants impaired in fruiting body formation. Therefore, the *A. fumigatus veA* gene represents a true functional orthologue of the *A. nidulans velvet* locus.

***veA* gene product is required for proper conidiation of *A. fumigatus*.** To assess any functional role of the *A. fumigatus-*



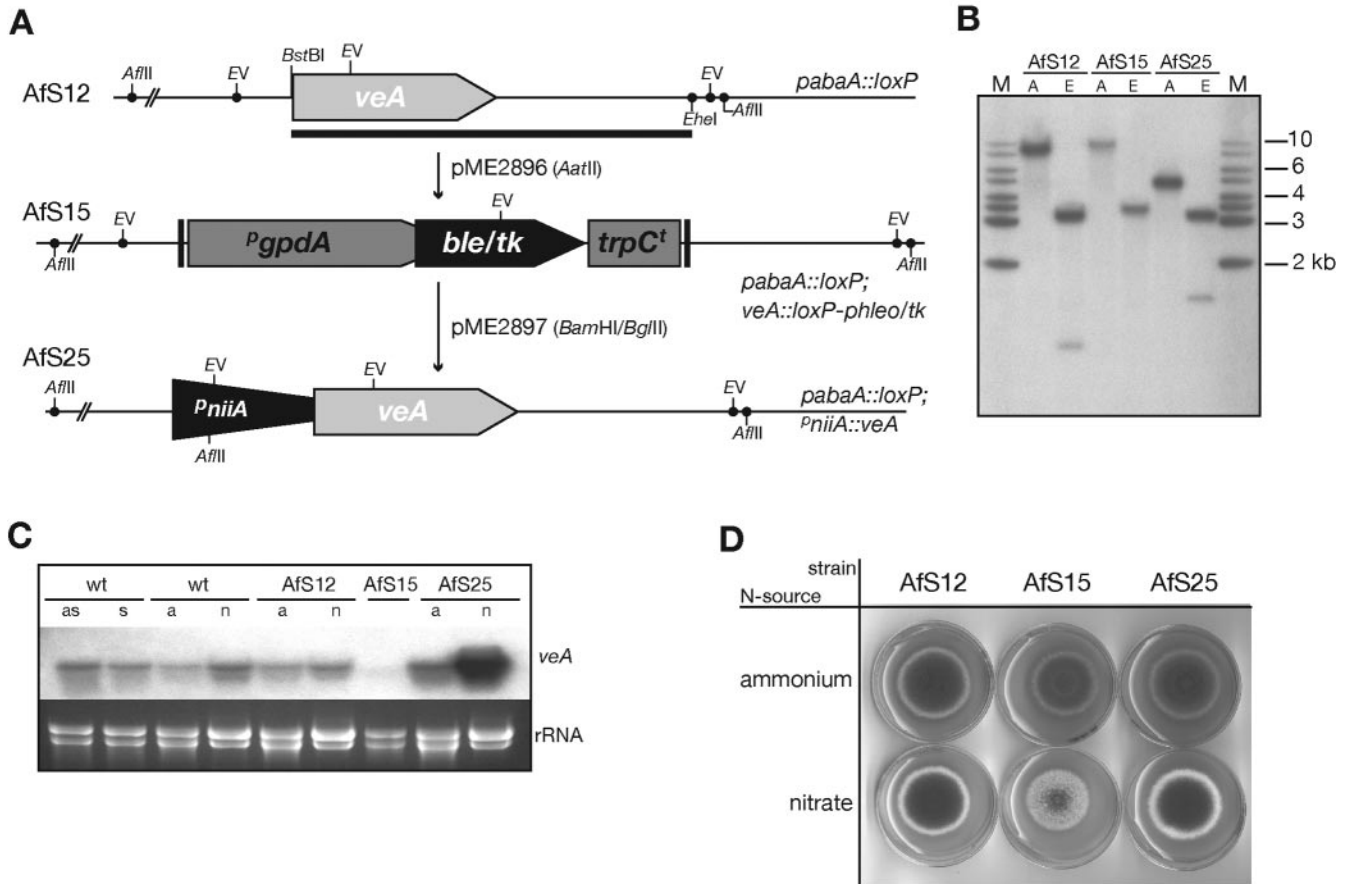


FIG. 4. Deletion and allelic exchange of the *A. fumigatus veA* gene. (A) Schematic representation of the *veA* open reading frame as well as the deleted locus before and after replacement of the *loxP-phleo*/*tk* marker module. Complete deletion of the *veA* coding sequence was achieved by transformation of the *pabaA* $\Delta$  strain AfS12 with a replacement cassette from pME2896. Exchange by the *niiA* promoter-driven allele from pME2897 could be selected by resistance of strain AfS25 to FUDR. Restriction sites are indicated; the horizontal bar indicates the position of the probe used in hybridization experiments. (B) Southern analysis of strains D141, AfS15, and AfS25. Corresponding genomic DNA was digested with *Afl*II (A) or *Eco*RV (E) and hybridization signals were detected employing a *veA*-specific probe as specified. Fragment lengths of the DNA size standard (M) are indicated. (C) Northern analysis of *A. fumigatus* strains for validation of the allelic exchange. Strains were propagated in the presence of nitrogen sources ammonium (a) or nitrate (n); additionally, induction of asexual (as) or sexual (s) differentiation as established for *A. nidulans* was applied for wild-type (wt) strain D141 by incubation with illumination or in the absence of light accompanied by restricted air exchange, respectively. Steady-state levels of the *veA* transcript are shown; ethidium bromide-stained rRNA signal served as a loading control. (D) Developmental characteristics of *A. fumigatus* strains expressing VeA at various levels. Equal amounts of spores were inoculated on medium supplemented with ammonium or nitrate as the sole nitrogen source and allowed to spread out at 37°C for 3 days.

among the transformants on this medium. Further inspection of one of these isolates (AfS25) by Southern hybridization confirmed homologous replacement of the *veA::loxP-phleo*/*tk* lesion by the *PniiA*-driven *veA* allele (Fig. 4B).

The strains generated were scrutinized by Northern analysis to estimate the steady-state levels of the *veA* transcript (Fig. 4C). For the *veA* $\Delta$  strain AfS15, no signal could be detected, confirming the deletion genotype of this strain. When propagated in liquid minimal medium containing the rich nitrogen source ammonium, the *veA* transcript is abundant in the wild-type strain D141 as well as in the *pabaA::loxP* descendant AfS12. When transferred onto solid ammonium-containing medium, a clear *veA* transcript could be detected for D141, irrespective of whether conditions that support asexual sporulation (aeration and illumination) or fruiting body formation (taping of plates and incubation in the dark) in *A. nidulans* were applied. Growing the fungus in the presence of the poor

nitrogen source nitrate resulted in *veA* transcript levels similar to those for ammonium-grown strains D141 and AfS12. For the reconstituted strain AfS25, clear regulation of *veA* expression was detected due to the nitrate-inducible *niiA* promoter that precedes the *veA* coding sequence: when propagated in the presence of ammonium; basal expression of the *veA* transcript could be monitored in Northern experiments, whereas induction of *veA* transcription was evident when nitrate was the sole source of nitrogen. Conclusively, overexpression of the *veA* gene under conditions of nitrate feeding had been accomplished by allelic replacement employing the versatile *loxP::phleo*/*tk* marker module.

Ultimately, the set of *A. fumigatus* strains generated were put on solid medium containing either ammonium or nitrate as the nitrogen source to assess any phenotypes linked to altered *veA* expression levels (Fig. 4D). Whereas no obvious growth phenotype could be observed for strain AfS25, which carries

the *PniiA::veA* allele, in comparison to its progenitor AfS12 on either type of medium, a clear reduction in sporulation capacities was observed for AfS15, which lacks the *veA* coding sequence. This diminution in the formation of asexual conidia was more pronounced in the presence of nitrate as the sole source of nitrogen. A rough estimation of spore numbers by counting the conidia from equal-sized plugs of mycelia yielded an approximately twofold decrease in spore quantity in the presence of ammonium and a  $\approx 10$ -fold reduction when nitrate had to be utilized by the fungus, each time compared to the wild-type strain grown on the same nitrogen source (data not shown). Moreover, this cut in sporulation capacity is not based on inappropriate supplementation with PABA or interference of the *pabaA::loxP* lesion with the *veA* deletion, as a strain deleted only of its *veA* locus (AfS13) displayed the same phenotype as strain AfS15 (not shown). This interesting phenotype indicates an influence of the nitrogen source on asexual sporulation of *A. fumigatus*, which seems to be balanced by the *veA* gene product. Moreover, our data indicate that sheer overexpression of the gene orthologous to *A. nidulans veA* is not sufficient to trigger any similar but cryptic developmental pathway in the asexual fungus *A. fumigatus*.

## DISCUSSION

In our aim to enhance the molecular biology of the opportunistic pathogen *A. fumigatus* we have designed and validated a novel marker system allowing gene replacement accompanied by marker rescue. Alternatively, based on its negative selection capacities, reconstitution or allelic exchange of target genes is facilitated by this marker module. The combination of several genetic functions—positive as well as negative selection together with acceptor sites for recombination—in a single marker module is a novel and applicable concept for the generation of genetic lesions in *A. fumigatus*. Because genetic markers are limited in this particular host, the concept of multiple marker usage, as proven before in *A. nidulans* (13, 35), was adapted in our studies.

The basic idea of recombinase-assisted marker rescue has been applied with great success in studying gene families in bakers' yeast (14), e.g., in the construction of *Saccharomyces cerevisiae* mutants impaired in hexose transport (60). Here, the highly redundant functions of more than 20 loci were eliminated by rounds of gene deletion and marker rescue, resulting in a strain completely devoid of any hexose transporter activity. In putting up the comprehensive Euroscarf (Frankfurt, Germany) yeast mutant collection, a recyclable blaster cassette was employed to facilitate downstream genetic manipulation of any deletion strain. With the genome sequence of *A. fumigatus* in hand, follow-up functional studies will have to include the generation of comprehensive knockout mutant collections, too.

The marker system developed may serve as a standard tool in these molecular studies for several reasons. (i) Dominance of the marker gene that replaces the gene of interest is important. Selection systems based on complementation of auxotrophies are generally hampered by the risk of incomplete complementation resulting in interfering phenotypes (3, 6). Especially in the in vivo situation of virulence studies, this may lead to biased results. (ii) The option of marker rescue is of

great advantage when studying gene families encoding redundant products. Furthermore, the genetic interactions of several gene lesions can be explored, a task that, due to the deficiency of sexual crossing techniques, has been prevented in *A. fumigatus* until now. (iii) The marker facilitates the homologous reconstitution of any targeted gene irrespective of an expressed strong phenotype that would allow direct selection. Clear reconstitution of a mutant strain is vital for phenotypic characterization and to prove that the nature of any observed phenotype is based solely on the lack of the gene locus and not on additional mutations.

However, we cannot exclude that expression of the viral thymine kinase might result in unwanted side effects that interfere with proper propagation in an infected host animal. On the other hand, the marker enables the direct comparison of a deletion strain carrying the complete marker module with a deletion strain in which the marker has been removed to leave a single *loxP* site, and future virulence studies will have to address this issue. Also, we seek to downsize the marker module by testing alternative promoter sequences that support expression of the resistance fusion protein at sufficient levels. Additionally, the necessity to aim at high-throughput processing when constructing deletion cassettes for the generation of a comprehensive mutant collection calls for automation of the cloning processes. Recombineering has evolved as the method of choice for purposes such as this, and we will explore the possibility of implementing this option in the novel marker module.

In our aim to provide a proof-of-concept we have deleted the *pabaA* locus of an *A. fumigatus* wild-type strain followed by marker excision. As indicated by other gene deletion experiments (our unpublished data), the low frequency of homologous recombination when deleting the *pabaA* locus is more likely due to the target locus than to the marker module. Precise excision of the marker module by the Cre/*loxP* system could be confirmed by sequencing of the genomic lesion, and the necessity for expression of the site-specific recombinase was demonstrated. As an alternative to plasmid-delivered Cre expression, electroporation of the recipient strain with purified, recombinant recombinase would facilitate the marker rescue procedure. Nevertheless, due to the likelihood of genetic rearrangements in applying this particular transformation method to *A. fumigatus* (20), we have not taken this into account. Furthermore, transformation of proteins via electroporation is not validated for aspergilli to our knowledge, so controlled and transient action of Cre appears more feasible by employing the expression plasmid.

When scoring the events of marker rescue, a frequency of 25% (5 out of 20) was observed. As this appears rather low, we have optimized the procedure by selecting primary pME2892 transformants on nitrate-containing medium and plating harvested conidia directly on FUDR plates without focusing on single isolates. Fast-sporulating clones can then clearly be identified on these plates and scrutinized further. Therefore, by screening a large number of descendants in this manner, segregates lacking the marker module can easily be isolated.

The resulting *pabaA::loxP* strain could facilitate molecular studies in this pathogen, as *Aspergillus* strains auxotrophic for the vitamin precursor PABA have been shown to be completely avirulent in several virulence testing models. This par-



ticular strain might serve as a safety strain when investigating basic features of *A. fumigatus* besides pathogenicity. One of the topics that have always been of interest is the apparent lack of any sexual cycle in the *A. fumigatus* lifestyle. Earlier studies stated a close relationship of the anamorph *A. fumigatus* and the teleomorph *Neosartorya fischeri*, but these organisms represent distinct species (22). So far, the conditions under which any cryptic sexual mode of propagation is executed by *A. fumigatus* have not been revealed.

Comparative genome studies have uncovered a variety of genetic features in this fungus that were characterized in other fungal species as required for mating, meiosis, or the formation of fruiting bodies, but their role in the life cycle of *A. fumigatus* remains enigmatic (16, 47, 58). One of these factors is the *veA* gene product. In this study we were able to demonstrate that the *veA* gene of the asexual fungus *A. fumigatus* is expressed and that it encodes a functional protein. By allelic exchange of the locus in *A. fumigatus* we were able to induce the expression of the *veA* gene in its original host under inappropriate conditions. Given the highly conserved genomic structure of the *Aspergillus veA* loci, the absence of any obvious phenotype when overexpressed in *A. fumigatus* was unexpected. However, this indicates that the genetic program of *A. nidulans* cleistothecium formation is not completely mirrored in the asexual relative and that a single factor might not be sufficient to trigger this cryptic developmental process in *A. fumigatus*.

Detailed examination of the fungal genomes of three aspergilli has uncovered the existence of two mating type idiomorphs, *MAT-1* and *MAT-2*, with either one being present in the *A. fumigatus* genome, whereas both are encoded by the genome of the homothallic species *A. nidulans* (58). Accordingly, the presence of both mating type loci in one vegetative thallus might be a strict prerequisite that determines mating ability in aspergilli to ensure proper communication between compatible mating partners. As a logical consequence, the sheer overexpression of positive regulators of sexual development might be insufficient to initiate the cellular program in the absence of activating upstream signals such as pheromone perception.

Furthermore, we could show that VeA is likely to function in the nitrogen metabolism of *A. fumigatus*, a role that has not been described for its *A. nidulans* counterpart. The reduced sporulation capacity of an *A. fumigatus veAΔ* strain in the presence of ammonium was accentuated by feeding nitrate. Chae and coworkers tested the sporulation capacity of their *A. nidulans veA* deletion strain DVAR1 on different media all containing the rich nitrogen source ammonium (32). However, the *A. nidulans veA* gene product was shown to influence the ratio of the  $\alpha$  and  $\beta$  transcripts synthesized from the *brlA* locus (31), a function that indicates an influence of the sexual effector on asexual sporulation. A *veA* deletion strain of the aflatoxin-producing fungus *Aspergillus parasiticus* was characterized to exhibit reduced conidial production, in particular when grown on its natural substrate peanut seed (10). This could specify a conserved role for the VeA factor in sporulation under adverse nutritional conditions. Comparative studies in the postgenomic era will have to address the nature of the numerous attributes that are directed by the *veA* gene product in different species of *Aspergillus*, as this conserved factor constitutes a key regulator of cellular processes in this highly relevant fungal genus.

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