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 (Emicellula) 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 aspergilloses 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...
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 *veA* allele. In *A. nidulans* the balance between asexual conidioaphore 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 *veA* strains this dependency on distinct environmental conditions is partly diminished, as these strains conidiate profusely in the absence of light but produce fruiting bodies in a delayed and reduced fashion (11). In its overall appearance, these *veA* mutant strains do not display the velvet 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 asexual 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 *faaA* gene, which encodes a fructosyl amine 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 DH5α (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 µg ml⁻¹. For recombinogenic engineering (recombinering) purposes (12), the bacterial strain K272 carrying the pKOBEG plasmid was grown in low-salt LB medium with 0.5% NaCl in the presence of 25 µg ml⁻¹ chloramphenicol. Minimal medium (0.52 g liter⁻¹ KCl, 0.52 g liter⁻¹ MgSO₄, 1.52 g liter⁻¹ KH₂PO₄, 0.1% trace element solution [29], pH 6.5) was used for growth of fungal strains, supplemented with appropriate amounts of 4-aminobenzoic acid (PABA, 1 µg ml⁻¹), phleomycin (30 µg ml⁻¹), 5-fluoro-2-deoxyuridine (FUDR, 100 µM), or pyrithiamine (0.1 µg 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 Gene Pulser 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 Red/amy 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 [α-³²P]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 Clustal-W/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 Xhol/NotI fragment from pME2256 in pBlueScript II KS (Stratagene). The plhceomycin blaster cassette pME2891 was constructed in five steps: an 0.4-kb ampicillin gene generated by PCR on pAn8-1 (49, GenBank accession number Z32751) with primer pair OLSK58 (EcoRI site, positions 3673 to 3695) and inserted into the EcoRV site of cloning phagemid pBluescript II KS, followed by insertion of a 1.1-kb niaD KpnI fragment (45) and the cin blaster cassette pME2891 was constructed in five steps: an 0.4-kb ampicillin gene was amplified from pSH47 (25) (accession number AF298782) with primer pair OLSK58 (EcoRI site, positions 3673 to 3695) and 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 gene (42) with oligonucleotides Sw66 (positions +4 to +22 of coding sequence) and Sw87 (positions +1131 to +1113). From the resulting plasmid a 1.5-kb Neol/XbaI fragment was released to replace a 1.1-kb Neol/XbaI fragment from pSH461 (GenBank accession number AF298782).

Next, the tptc 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 (accession number AF298782) with primer pair OLSK58 (EcoRI site, positions 3673 to 3655) and inserted into the EcoRV site of pME2890, which carries the *A. nidulans niaA* intergenic region as a 1.2-kb KpnI fragment (45) and the *niaD* 5' region (accession number M85291) as an XbaI/SpeI ampicillin module from genomic DNA via *SsiI* (positions 3303 to 3325) and *SstI* (positions 3558 to 3565). The resulting *niaD:cre* module was amplified by PCR and inserted into the SmaI site of the autonomously replicating Aspergillus plasmid pTRII 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 recombinering linearized pME2893 with the PCR product of primers *Svi*160 and *Svi*161 on template pME2891 (12, 37). For construction of pME2896, the 4.5-kb Hpal/HindIII fragment of pME2893 was ligated to the Csp451/HindIII backbone of pME2895 to yield the *veA*:loxP-phleo/tk deletion cassette. The

TABLE 1. Strains used in this study

| Strain | Genotype | Reference |
|--------|----------|-----------|
| D141   | *A. fumigatus* wild type, clinical isolate | 50 |
| AIS11  | *pabaA*:loxP-phleo/tk | This study |
| AIS12  | *pabaA*:loxP | This study |
| AIS13  | *veA*:loxP-phleo/tk | This study |
| AIS15  | *pabaA*:loxP *veA*:loxP | This study |
| AIS25  | *pabaA*:loxP *niiA*:loxP | This study |
pME2897 reconstitution allele to exchange the endogenous \textit{veA} promoter sequence for the \textit{A. fumigatus} \textit{niaD} \textit{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 \textit{niaD} coding sequence) and Sv234 (BstBI, positions –10 to –30) from the genomic DNA of strain D141 into the unique BstBI site of plasmid pBluescript II KS derivative carrying BgIII site in polylinker Collection of AG Braus

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 \textit{Aspergillus fumigatus}. The molecular biology of the pathogen \textit{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 \textit{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-fluorodeoxyuridine (FUDR). To allow constitutive expression in the fungal host, this sequence stretch was positioned between the \textit{gpdA} promoter sequence and \textit{trpC} terminator region of \textit{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 \textit{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 \textit{loxP}-specific recombinase, an expression module placing the encoding \textit{cre} gene between the \textit{A. nidulans} \textit{niaD} \textit{5'} and \textit{3'} regulatory sequences

| 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 CCC CAT C-3' |
| Sv88………………..| 5’-TTC ATT ACT TCT AGA CGG GTT CG-3' |
| Sv89………………..| 5’-AAG AGG TAT TAG TCT ACA GTG-3' |
| Sv95………………..| 5’-GCA TCT CCA GGC ACC TCT CAT CTA GGC CAT TAG TGG-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-3’ |
| Sv161……………….| 5’-TGA AGT ATT CAC CTC GAC GTC CAA AAC ATC ATC CAC AAC AAG AAA GAA GAG ATG CTT GCA GCT GAA GTC TGC TAC GC-3' |
| Sv231……………….| 5’-TAC ATT CGA AGA GTA CTA CAG CAT CTA ATG TCG GTA TAG-3' |
| Sv232……………….| 5’-ATC GTG CGA AGT CTC AAC GGC 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 CCT CTA GGC GC-3' |

TABLE 2. Plasmid constructs employed in this study

| Plasmid | Description | Reference |
|---------|-------------|-----------|
| pBluescript II KS | General cloning plasmid (bla, multiple cloning site) | Stratagene |
| pUG6 | kanMX marker module (bla loxP+TEF2-kan'loxP) | 25 |
| pSH47 | Yeast Cre expression plasmid (PGAL1::cre URA3 ARS/CEN) | 25 |
| pAN7-1 | Aspergillus marker cassette conferring resistance to hygromycin [pgdA::hph::trpC'] | 49 |
| pAN8-1 | Aspergillus marker cassette conferring resistance to phleomycin [pgdA::ble::trpC'] | 49 |
| pPTRH | Autonomously replicating Aspergillus plasmid (pAA AM1 bla) | Takara |
| pME2256 | Yeast plasmid pRS316 (URA3, ARS/CEN) with BgIII site in polylinker | Collection of AG Braus |
| pME2889 | pBluescript II KS derivative carrying BgIII site in polylinker | This study |
| pME2890 | A. nidulans niaD::niaD' expression module in pBluescript II KS | This study |
| pME2891 | loxP-phleo/tk blaster (loxP+pgdA::ble/HSV1 tk::trpC+loxP) | This study |
| pME2892 | Cre expression module in pPTRH (A. nidulans niaD::cre pAA AM1) | This study |
| pME2893 | pabaA genomic locus as 9.0-kb BamHI/SacI fragment in pBluescript II KS | This study |
| pME2894 | pabaA deletion cassette (pabaA::loxP-phleo/tk) | This study |
| pME2895 | veA genomic locus as 6.7-kb BglII/SmaI fragment in pME2889 (BglII/SmaI) | This study |
| pME2896 | veA deletion cassette (veA::loxP-phleo/tk) | This study |
| pME2897 | veA reconstitution allele for promoter insertion (niaA::veA) | This study |

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 CCC CAT C-3' |
| Sv88………………..| 5’-TTC ATT ACT TCT AGA CGG GTT CG-3' |
| Sv89………………..| 5’-AAG AGG TAT TAG TCT ACA GTG-3' |
| Sv95………………..| 5’-GCA TCT CCA GGC ACC TCT CAT CTA GGC CAT TAG TGG-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-3’ |
| Sv161……………….| 5’-TGA AGT ATT CAC CTC GAC GTC CAA AAC ATC ATC CAC AAC AAG AAA GAA GAG ATG CTT GCA GCT GAA GTC TGC TAC GC-3' |
| Sv231……………….| 5’-TAC ATT CGA AGA GTA CTA CAG CAT CTA ATG TCG GTA TAG-3' |
| Sv232……………….| 5’-ATC GTG CGA AGT CTC AAC GGC 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 CCT CTA GGC GC-3' |
was constructed. This \textit{niaD::cre} component was cloned into plasmid pPTRII, suitable for autonomous replication in \textit{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 \textit{Aspergillus fumigatus} in addition to homologous reconstitution of deletion mutant strains (for a detailed protocol outline, see our website at http://wwwuser.gwdg.de/~molmbio/organigr.htm).

\textbf{Generation of an \textit{A. fumigatus} pabaA::loxP strain.} In order to validate our marker system we targeted the \textit{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 \textit{Aspergillus} mutant strains have been shown in earlier studies to be dramatically impaired in their virulence characteristics, and the \textit{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 recombineering approach, which is genetic engineering based on homologous recombination in an \textit{E. coli} host strain expressing phage-derived proteins (44). For that purpose, the \textit{loxP}-\textit{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 \textit{pabaA} locus. Cotransformation of linearized plasmid pME2895 with the amplicon into the arabinose-induced \textit{E. coli} host strain KS272/pKOBEG (12) yielded the recombined construct pME2894, in which the \textit{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 \textit{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 pyridoxamine. To induce \textit{niaD}-driven expression of the \textit{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 \textit{loxP} sites. Furthermore, the \textit{pabaA::loxP} lesion from two FUDR isolates after Cre expression was amplified from the genome and sequenced to validate precise recombination between the \textit{loxP} sites. Identical sequences from both isolates demonstrated the presence of one \textit{loxP} site flanked by 5’ and 3’ \textit{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 pyridoxamine. All of these descendants were sensitive to pyridoxamine, 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 \textit{A. fumigatus} by the novel dominant marker module and successfully rescued the genetic marker after precise excision mediated by Cre/\textit{loxP} recombination. The resulting \textit{pabaA::loxP} strain can serve as the recipient in any subsequent gene targeting procedure.
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 veA gene restored the phenotypes originating from the genetic lesion (not shown): these reconstituted isolates did profoundly 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-
encoded VeA in the life style of this asexual fungus, we aimed at inactivation of the encoding gene (Fig. 4A). For this purpose, regions encompassing 2.7 kb of the 5'/H11032 and 2.2 kb of the 3'/H11032 region of the target locus were cloned to border the loxP-phleo/tk marker. The resulting cassette from pME2896 was then transformed into strain AfS12 with its loxP genetic background, and selection was carried out on PABA- as well as phleomycin-containing medium. Inspection of the transformant pool by Southern analysis revealed several isolates in which the endogenous veA gene had been replaced by the dominant marker (Fig. 4B) to yield strain AfS15.

To demonstrate and exploit the negative selection capacities of the novel marker module, we further intended to reconstitute this strain by integration of an alternative veA construct at the homologous gene locus. Therefore, the niiA promoter sequence of *A. fumigatus* was inserted upstream of the presumed veA start codon to result in an allele whose expression can be adjusted by the added nitrogen source (1). This reconstitution cassette, covering the same flanking regions as pME2896, was transformed into the pabaA::loxP veA/H9004 deletion mutant, and transformants were screened for reconstituted strains based on their growth phenotype on FUDR-containing medium. Whereas the recipient strain AfS15 grew poorly on FUDR control plates, several single sporulating colonies were visible.
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 veAΔ 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 nitrate as 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

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**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 pabaAΔ 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 AflII (A) or EcoRV (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.
the p\textsuperscript{*}nidA::veA allele, in comparison to its progenitor AF12 on either type of medium, a clear reduction in sporulation capacities was observed in AF15, 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 (AF13) displayed the same phenotype as strain AF15 (not shown). This interesting phenotype indicates an influence of the nitrogen source on asexual sporulation of \textit{A. fumigatus}, which seems to be balanced by the veA gene product. Moreover, our data indicate that sheer overexpression of the gene orthologous to \textit{A. nidulans} veA is not sufficient to trigger any similar but cryptic developmental pathway in the asexual fungus \textit{A. fumigatus}.

**DISCUSSION**

In our aim to enhance the molecular biology of the opportunistic pathogen \textit{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 \textit{A. fumigatus}. Because genetic markers are limited in this particular host, the concept of multiple marker usage, as proven before in \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 *veA* gene 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 sheen 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 DVR1 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 α and β transcripts synthesized from the *b1R* 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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