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TECHNICAL NOTE

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Disruption of the *Aspergillus fumigatus argB* gene using a novel in vitro transposon-based mutagenesis approach

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Abstract We disrupted the *Aspergillus fumigatus argB* gene, encoding ornithine transcarbamylase, using a novel in vitro transposon-based mutagenesis approach. This approach utilizes a modified transposon containing the *Neurospora crassa pyr4* gene, which is randomly inserted in vitro into a target sequence of interest. Clones in which the gene of interest has been disrupted are identified by PCR and used to transform a *pyrG*-deficient strain of *A. fumigatus*. Using this approach, we obtained arginine auxotrophs of *A. fumigatus*. Full characterization of the *argB* insertion was performed by Southern blot analysis. These strains can be supplemented by addition of arginine into the culture medium and can be fully rescued to arginine prototrophy by transformation with the intact *A. fumigatus argB* gene.

Keywords *Aspergillus fumigatus* · Arginine auxotroph · Transformation · Transposon

Introduction

Aspergillus fumigatus is a saprophytic filamentous fungus which is ubiquitous in nature. In recent years, it has become an important pathogen in immunocompromised patients. *A. fumigatus* is capable of causing a wide range of diseases, invasive aspergillosis being the most serious (Latge 1999), with mortality rates reaching 30–90% despite aggressive antifungal treatment (Denning 1998). During the past 20 years, with the number of immunocompromised patients growing dramatically, the incidence of invasive aspergillosis has increased markedly, emphasizing the need for a better understanding of the pathogenicity of *A. fumigatus*.

Molecular biology provides essential tools for investigating the mechanisms underlying fungal virulence and developing novel therapeutic drugs. A prerequisite to study *A. fumigatus* pathogenesis at the molecular level is the development of efficient gene transfer systems. This allows gene manipulation and the identification of genes encoding potential virulence factors. Conventional transformation techniques, such as protoplast transformation and electroporation of germinating conidia, have been applied successfully for *A. fumigatus* (Brakhage and Langfelder 2002). Two approaches for selectable transformation markers have been used for *A. fumigatus*: dominant selectable markers such as hygromycin B (Punt et al. 1987; Punt and van den Hondel 1992; Monod et al. 1993; Tang et al. 1993) and phleomycin/bleomycin (Smith et al. 1994; Mellado et al. 1996) and auxotrophic markers such as *pyrG*, which encodes orotidine-5′-monophosphate (OMP)-decarboxylase (d'Enfert 1996). *pyrG* mutants are characterized by their auxotrophy to uracil and resistance to 5-fluoro-orotic acid (Boeke et al. 1984). d'Enfert (1996) was the first to apply the homologous transformation system (*pyrG*-blaster) based on *pyrG* to disrupt *A. fumigatus* genes. The *pyrG*-blaster system is composed of a disruption cassette which includes the *A. niger pyrG* gene flanked by a direct repeat which encodes the neomycin phosphotransferase of transposon, Tn5. Mutants containing genomic insertions in the specified gene were selected in the presence of uracil and 5-fluoro-orotic acid. This selection resulted in the excision of *pyrG*, while producing neomycin-inserted mutants obtained by recombination between the two elements of neomycin. Recently, another homologous transformation system based on the *sC* gene (encoding ATP sulfurylase) was developed (De Lucas et al. 2001). Using this system, mutants of *A. fumigatus* were isolated by their resistance to selenate.

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Transformation systems using auxotrophic markers enjoy several advantages: they permit clear identification of transformants (Weidner et al. 2001; Brackhage and Langfelder 2002), allow targeted integration of the desired genetic construct at a specific locus (Weidner et al. 1998) and allow multiple gene deletions (d'Enfert 1996; De Lucas et al. 2001). As new sequence data from the recently completed *A. fumigatus* sequencing project becomes available, deletion of specific genes of *A. fumigatus* to generate additional auxotrophic marker strains can be accomplished.

In this study, we describe a fast and simple technique which combines the *pyr4* gene of *Neurospora crassa* (encoding OMP-decarboxylase) and the transprimer 1 transposon of the GPS1 in vitro transposon mutagenesis system to generate *argB* mutants of *A. fumigatus* deficient in ornithine transcarbamylase (OTCase). This technique can be used to rapidly delete any gene of interest in the *A. fumigatus* genome.

Materials and methods

Strains and culture conditions

A. fumigatus strain AF293.1, a derivative of strain AF293 originally isolated at autopsy from a patient with invasive pulmonary aspergillosis, was used throughout this study (Osherov et al. 2001). This strain is deficient in *pyrG* (encoding OMP-decarboxylase) and, consequently, is auxotrophic for uridine/uracil. YAG UU medium, which consists of 0.5% yeast extract, 1% glucose, 10 mM $MgCl_2$, 1.5% agar (when needed), 10 mM uracil and 5 mM uridine, supplemented with trace elements and vitamins (Bainbridge 1971), was used for growth. Conidia were harvested in 0.2% Tween 80, resuspended in double-distilled water (DDW) and counted in a hemacytometer.

AF293.1 transformants were grown in YAS CM plates (containing 0.5% yeast extract, 1% glucose, 10 mM $MgSO_4$, 0.2 M sucrose, 1.5% agar, trace elements, vitamins; Bainbridge 1971). For selection of *A. fumigatus* clones deficient in *argB* (encoding ornithine transcarbamylase), minimal medium (MM) supplemented with 5 mM arginine was used. MM was composed of 70 mM $NaNO_3$, 1% glucose, 12 mM KPO_4 pH 6.8, 4 mM $MgSO_4$, 7 mM KCl, trace elements and 1.5% agarose. When mentioned, 10 mM uracil and 5 mM uridine were also added.

Escherichia coli strains BW23474 (CGSC 7838; Metcalfe et al. 1994) and DH10B (Invitrogen Corp., Carlsbad, Calif.), used for replication of pGPS1 and the T/A cloning vector, respectively, were grown in LB medium at 37 °C with shaking at 250 rpm. When required, 100 μg ampicillin/ml and 25 μg kanamycin/ml were added.

A. fumigatus genomic DNA purification

Freshly-harvested conidia (5×10^6/ml) were grown for 20 h at 37 °C in 30 ml of liquid YAG UU in 10 cm plates. Hyphae were harvested on miracloth, rinsed once with DDW, pressed to remove water and transferred to an Eppendorf tube. Following freezing in liquid nitrogen and lyophilization, the dried mycelium was ground using a 1-ml sterile blue tip. An equivalent of 50–100 μl of mycelial powder was transferred to a new tube and an equal volume of glass beads (150–212 μm, acid washed; Sigma, St. Louis, Mo.) was added. The mix was ground again for 2–3 min to produce a fine fungal powder. Then, 0.7 ml of prewarmed lysis solution (10 mM Tris-HCl, 100 mM EDTA, 0.5% SDS, pH 8) were added and the mix was vortexed for 2 min and incubated at 65 °C for 30 min. Chromosomal DNA was extracted twice with phenol and then twice with phenol:chloroform. The supernatant containing DNA was subjected to digestion by RNAse (10 μg/ml) at 37 °C for 30 min. Chromosomal DNA was extracted again once with phenol:chloroform and once with chloroform. To precipitate the DNA, 0.1 vol. of 3 M sodium acetate (pH 5.2) and 3.0 vol. of ethanol were added. The pellet containing the genomic DNA was washed once with 70% ethanol, dissolved in 30 μl of DDW and stored at −20 °C until used.

PCR amplification of the *argB*-containing genomic fragment

A 4,223-bp DNA fragment flanking the *A. fumigatus argB* (Afu*argB*) homologue was generated by PCR, using the Expand high fidelity PCR system (Roche Diagnostic, Penzberg, Germany) and the following primers: AfuArgB4223 forward primer (5′-AT*GGCGCGCC*GACACGTCAAACGCCAATG-3′) and AfuArgB4223 reverse primer (5′-ATC*GGCGCGCC*AGATGAGTCCGGATGCTTTG-3′). These primers were designed to contain an *Asc*I restriction site at their 5′ end (marked in italics). The following PCR conditions were used as recommended by the manufacturer, with minor modifications: denaturation of the chromosomal DNA template (0.5 μg) at 94 °C for 2 min, followed by ten cycles of denaturation at 94 °C for 10 s, annealing at 60 °C for 30 s and elongation at 68 °C for 3 min, with 20 additional cycles in which the elongation was performed at 68 °C for 3 min and 20 s were added to each successive cycle. A final step of incubation at 68 °C for 15 min was added to facilitate cloning of the PCR fragment into pGEM T/A cloning vector (Promega Corp., Madison, Wis.).

Insertional inactivation of *A. fumigatus argB*

Inactivation of *argB* was performed using the GPS-1 genome-priming system (New England Biolabs, Beverly, Mass.). However, to facilitate later selection of *A. fumigatus argB* mutants, the pGPS1 transprimer 1 transposon region of the plasmid was modified to include the *Neurospora crassa pyr4* gene (encoding OMP-decarboxylase). The *pyr4* gene was released from the pBS-SK-*pyr4* plasmid containing a 2,000-bp genomic fragment of *pyr4* (Waring 1989) by cleavage with *Xba*I and was then ligated to pGPS1 previously cut with *Spe*I. This resulted in the integration of the *pyr4* gene at the 3′ end of the transprimer 1 transposon region of the pGPS1 plasmid. To obtain sufficient amounts of the pGPS1 plasmid carrying *pyr4*, the recombinant plasmid was introduced into *E. coli* strain BW23474 (CGSC 7838) by electroporation and purified using the Plasmid midi kit (Qiagen, Valencia, Calif.). The PCR-amplified 4,223-bp *arg*B-containing fragment was gel-purified using the Wizard SV gel and clean-up system (Promega Corp.) and cloned into the pGEM T/A cloning vector, as instructed by the manufacturer (Promega Corp.). This vector was then used as a target for the transprimer 1–*pyr4* transposon. Transposition was performed according to the manufacturer's instructions (New England Biolabs). pGEM T/A carrying the transprimer 1–*pyr4* transposon was cloned into *E. coli* DH10B strain. Clones carrying transposon-disrupted *argB* plasmids were identified by PCR using Taq polymerase (Fermentas UAB, Vilnius, Lithuania), AfuArgB1206fp forward primer (5′-TACAGCGTCGTTGGGTTCTC-3′) and AfuArgB1206rp reverse primer (5′-ATTTGGTTTCGGCTTGCTTT-3′). These primers allowed amplification of a 1,206-bp fragment containing the *A. fumigatus argB* gene. For verification of the transposon-disrupted *argB* clones, the transprimer 1 inverted primers supplied in the GPS-1 kit were used: primer N (5′-GTTTAAGACTTTATTGTCCG-3′), Primer S (5′-GTTCCCAACTATTTTGTCCG-3′). The transprimer 1–*pyr4* disrupted *argB* fragment was released by cleavage with *Asc*I, purified with Wizard SV gel and clean-up system and used for transformation of *A. fumigatus* AF293.1.

A. fumigatus transformation

Transformation was performed as described by Osherov et al. (2000), except for the following modifications:

1. Prior to transformation, conidial protoplasts were resuspended in 0.5 ml of 50 mM $CaCl_2$, 0.6 M KCl, 10 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol and incubated overnight at 4 °C.
2. Following transformation with *Asc*I-cut plasmid DNA and plating on YAS CM plates, the plates were incubated overnight at room temperature and then for 2 days at 37 °C. These modifications significantly (2- to 5-fold) increased the efficiency of transformation.

For transformation of the *A. fumigatus argB*-deleted mutants, the same procedure was used. However, MM medium supplemented with 0.6 M KCl was used for growth of the transformants throughout the whole procedure.

Nucleic acid manipulation

DNA blotting, Southern blot analysis and electroporation of *E. coli* were performed as described by Sambrook and Russell (2001). For Southern blot analysis, 5 µg of fungal DNA were digested with *Eco*RI and run on a 1% agarose gel. Cleaved DNA was transferred to Nytran-N Nylon membranes (Schleicher & Schuell, Germany) by capillary reaction (Sambrook and Russell 2001) and hybridized with *A. fumigatus argB* or *pyr4* probes at 65 °C. *A. fumigatus argB* and *N. crassa pyr4* probes were prepared by the random priming method, using α^{32}[dCTP] as described by Sambrook and Russell (2001).

Results

Identification of the *A. fumigatus argB* homologue, Afu*argB*

The *argB* gene of *A. nidulans* was used to search for the *A. fumigatus argB* homologue, using the blast program at the TIGR *A. fumigatus* fungal database (http://www.tigr.org/tdb/fungal). This search revealed that only one *argB* homologue (Afu*argB*) exists in the *A. fumigatus* genome. Multiple protein alignments showed that Afu*argB* encodes OTCase (Fig. 1).

Construction of the *A. fumigatus argB* knockout vector

The insertional inactivation strategy for *argB* is depicted in Fig. 2. A 4,223-bp fragment flanking the *argB* gene

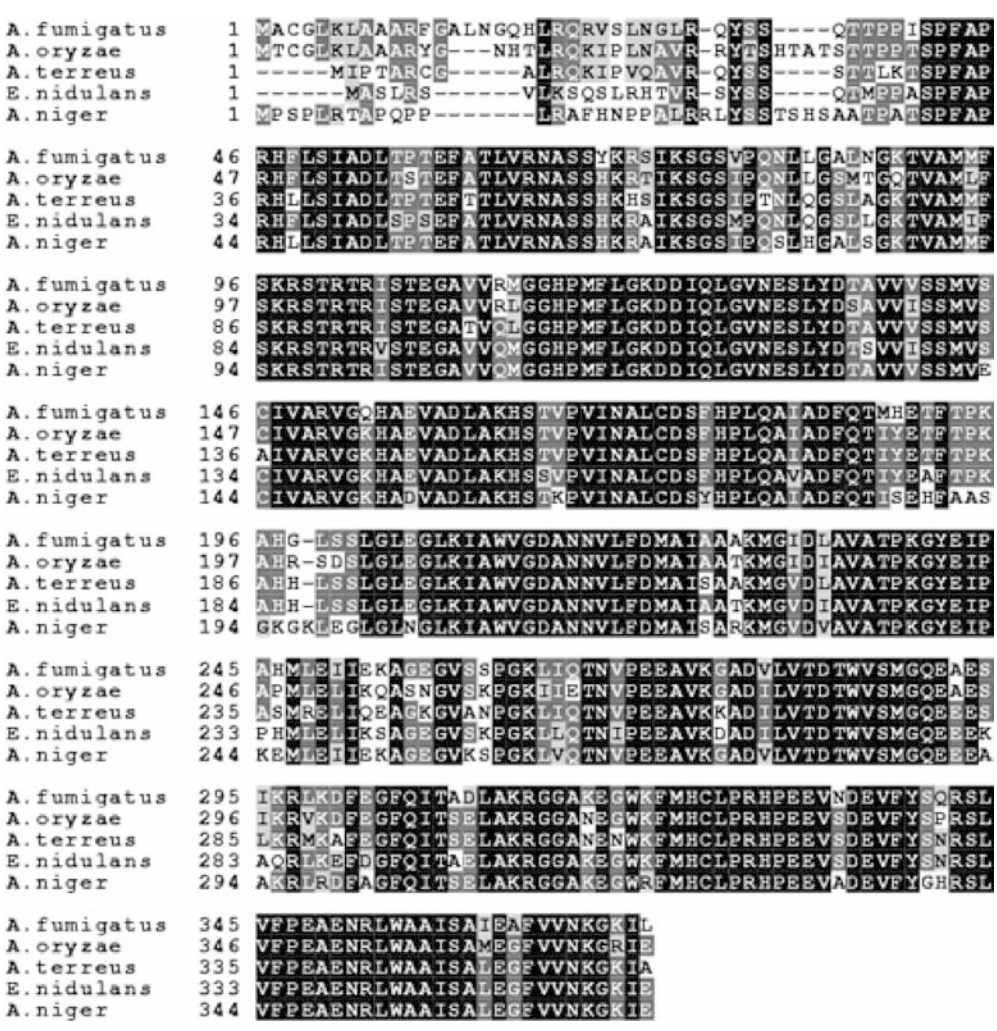

Fig. 1 Amino acid comparison of *Aspergillus fumigatus* ornithine transcarbamylase (OTCase) with *A. oryzae* (GenBank accession number BAA35119), *A. terreus* (Q00291), *Emericella nidulans* (AAA50816) and *A. niger* (P11066) OTCases. The Clustal W 1.8 program was used for the alignments. A *dash* indicates a gap in the sequence. A *black background* indicates that the sequence is identical among all the aligned species, *dark gray* indicates it is identical in three species and *light gray* indicates a conserved sequence

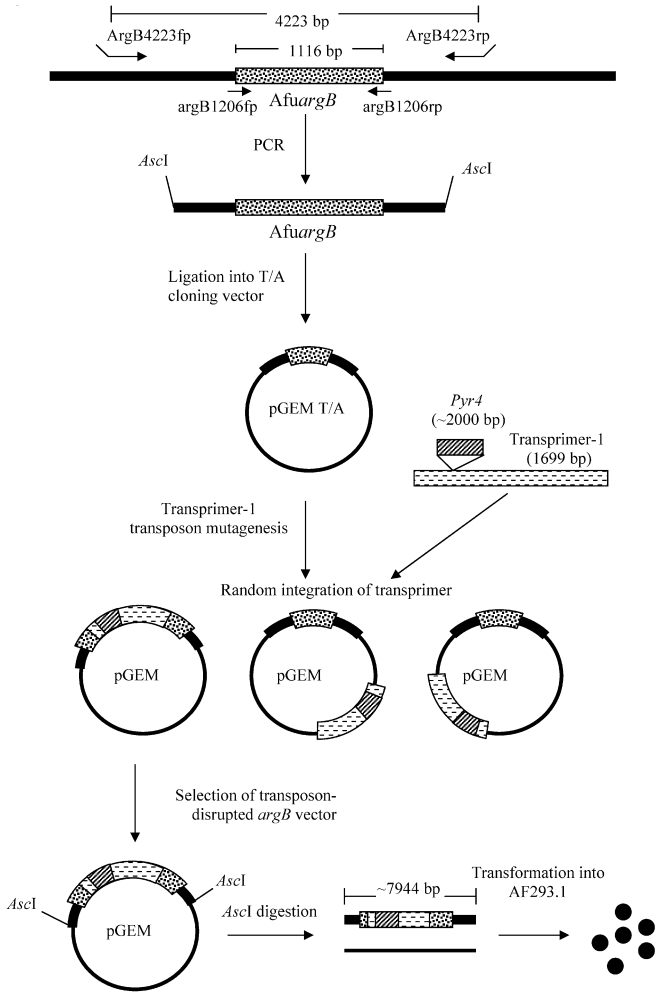

Fig. 2 Scheme for the in vitro transposon mutagenesis approach used to create the targeted disruption of the *A. fumigatus argB* gene (see Materials and methods for a detailed description)

was amplified from *A. fumigatus* genomic DNA by high-fidelity PCR and cloned into a T/A cloning vector. This vector was then used as a target for the *pyr4*-containing transprimer 1 transposon (see Materials and methods). Randomly transposon-integrated vectors were cloned into *E. coli*. Clones containing the transposon were selected on LB plates containing kanamycin. To identify *argB*-disrupted clones, plasmid DNA was purified from 45 clones and used as a template for PCR with Taq polymerase and primers flanking the *argB* gene. A PCR product of 1,206 bp was predicted from clones carrying intact *argB*, whereas clones containing transprimer 1–*pyr4* disrupted *argB* were expected to give a PCR product of 4,905 bp (Fig. 3A). Because of the short extension time (1 min) and the size of the inserted transposon (3,699 bp), no PCR product was obtained from the *argB*-disrupted clones. PCR analysis revealed that five of the 45 clones showed no product, which suggests that they carried plasmids containing disrupted *argB* (Fig. 3A, lanes 16, 33, 34, 37, 40). To map the integration site, each of these plasmids was further analyzed by PCR, using the forward and reverse primers of *argB* with the inverted N and S primers of the transposon. Four of these clones produced a PCR product with the primer couples of: (a) the forward primer of *argB* and primer N and (b) the reverse primer of *argB* and primer S of the transposon, whereas one clone produced a PCR product with the primer couples of: (c) the forward primer of *argB* and primer S and (d) the reverse primer of *argB* and primer N of the transposon (Fig. 3B). The sizes of these fragments indicated that the transprimer 1–*pyr4* transposon was integrated in either of two orientations and at different sites in the *argB* gene in each clone (Fig. 3C). One clone (C1), in which the *pyr4*-containing transprimer 1 transposon had integrated 200 bp from the 5′ end of *argB*, was chosen for transformation of *A. fumigatus* AF293.1.

Isolation of insertion-inactivated *argB* mutants of *A. fumigatus*

To facilitate double recombination, the T/A cloning vector carrying the disrupted *argB* was cleaved with *Asc*I prior to transformation, resulting in the release of the 7,944-bp transprimer 1–*pyr4* disrupted *argB* fragment (Fig. 2). Following transformation into the *pyrG*$^{-}$ mutant *A. fumigatus* AF293.1, 41 transformants of *A. fumigatus* were obtained in which *pyrG* deficiency was complemented with *pyr4*. Assuming a correct integration rate of 10–15% (d'Enfert et al. 1999), this is expected to yield approximately 4–6 *argB*-inactivated transformants. To select for transformants in which the native *A. fumigatus argB* was replaced by a double homologous recombination with the transposon-disrupted *argB*, conidia from all 41 transformants were grown in MM in the presence and absence of arginine. Six clones, designated AF293.1/1–AF293.1/6, were able to grow only in MM supplemented with arginine. Five of these clones are shown in Fig. 4.

Southern blot and complementation analysis

To verify the insertional inactivation of *A. fumigatus argB* in the AF293.1/1–AF293.1/6 strains by homologous recombination, genomic DNA of the parent and mutant strains was purified, cleaved with *Eco*RI and hybridized with the *A. fumigatus argB* and *N. crassa pyr4* probes. *Eco*RI cuts 972 bp upstream and 2,998 bp downstream of *argB*, but it does not cut within *argB* or transprimer 1–*pyr4* (3,699 bp; Fig. 5A). Therefore, a band of approximately 8,785 bp is expected when hybridizing either probe with the AF293.1/1–AF293.1/6 mutants, compared with a band of 5,086 bp for the control parental genomic DNA (Fig. 5A). Indeed, Southern blot analysis revealed the existence of bands of the expected sizes (Fig. 5B, C). In order to rule out the occurrence of a mutation other than the *argB* mutation, which could account for the *argB*$^{-}$ phenotype, all six

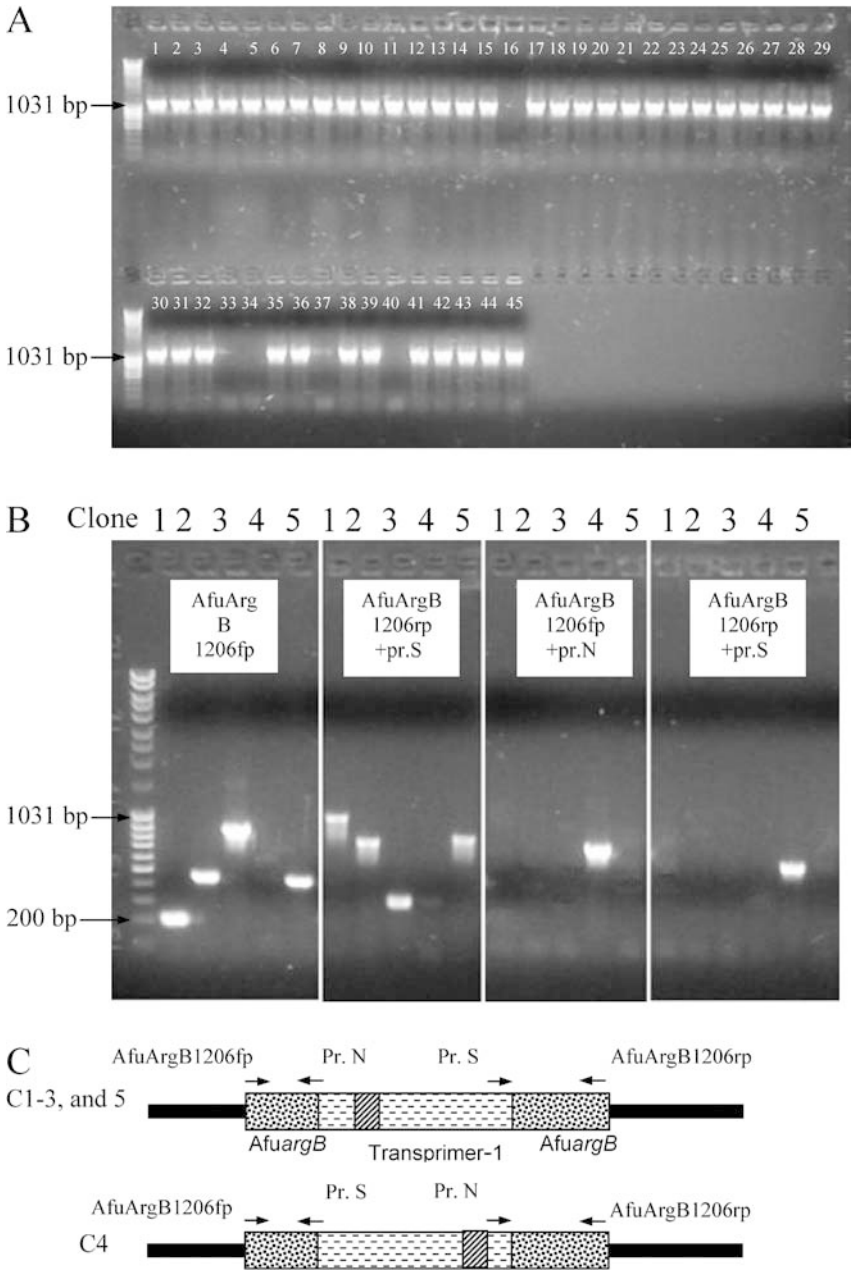

Fig. 3A–C Identification of the *argB* knockout vector derived by in vitro transposon mutagenesis. **A** PCR amplification using the Afu*argB*1206fp forward primer and Afu*argB*1206rp reverse primer of plasmid DNA purified from independent clones containing randomly inserted transposon in the *argB* plasmid. The expected 1,206-bp PCR product is lacking in clones 1–5 (*lanes 16*, *33*, *34*, *37*, *40*) containing an insertion into *argB*. **B** Mapping of the *ArgB* insertion site in clones 1–5, using the Afu*argB*1206fp forward primer and Afu*argB*1206rp reverse primer in combination with transposon primers S and N. **C** Maps outlining the different insertions in clones 1–5 (*C1*, etc), based on the results from part **B**

mutants were transformed with the T/A cloning vector carrying the intact 4,223-bp *argB*-containing fragment and tested for complementation of the mutated *argB*. All six mutants underwent transformation with the vector at transformation efficiencies comparable with that of strain AF293.1 transformed with the T/A cloning vector carrying the transprimer 1–*pyr4* disrupted *argB* fragment and all were converted to arginine heterotrophy (data not shown).

Discussion

Knockout of genes by insertional mutagenesis requires efficient selectable markers for transformation. Two selection marker strategies are available for *A. fumigatus*: auxotrophic selectable markers (d'Enfert 1996; De Lucas et al. 2001) and dominant selectable markers (Punt et al. 1987; Punt and van den Hondel 1992; Smith et al. 1994; Mellado et al. 1996). For both of these strategies to be implemented, long fragments (>1 kb) flanking the gene of interest are needed to allow efficient homologous recombination (>10%; d'Enfert et al. 1999). The fragment must first be cloned from a genomic DNA library, mapped for suitable excision from the vector and for insertion of a selectable marker. Here, we have established a fast and simple knockout gene strategy, based on a long PCR technique combined with the GPS-1 genome-priming system and the *pyr4* transformation selectable marker. This system is shown to

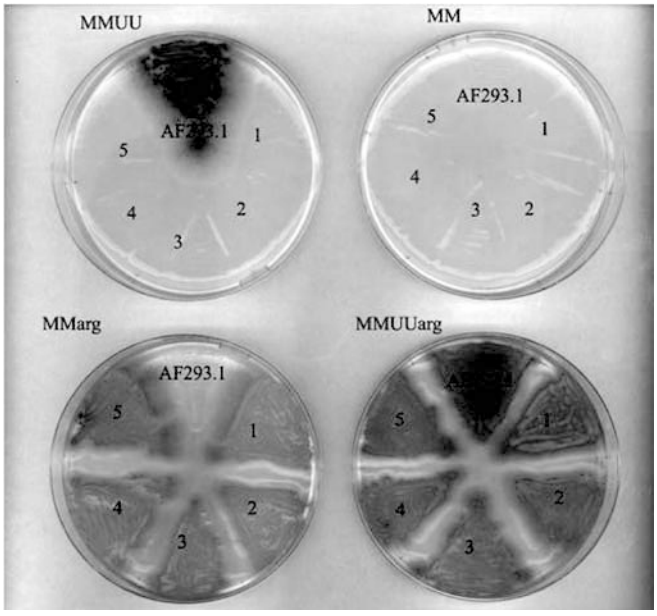

Fig. 4 Growth analysis of the *A. fumigatus* arginine auxotroph strains. The growth of five independent transformants was compared with that of the parental AF293.1 strain. *MM* Minimal medium agar plates without arginine or uracil/uridine, *MMUU* MM with uracil/uridine, *MMarg* MM with arginine, *MMUUarg* MM with arginine and uracil/uridine. Plates were incubated for 48 h at 37 °C

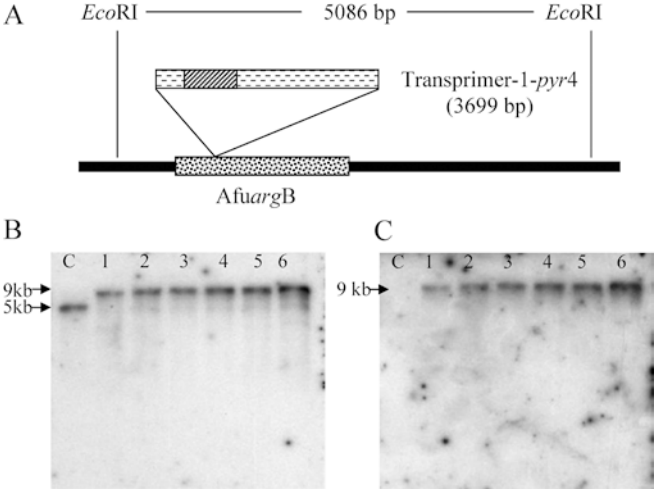

Fig. 5A–C Disruption of the *A. fumigatus argB* gene. Gene disruption was detected by Southern blot analysis. Genomic DNA from the *A. fumigatus pyrG*-deficient strain AF293.1 (*lane C*) and independent transformants (*lanes 1–6*) was digested with *Eco*RI. **A** Map of the genomic locus of *A. fumigatus argB*, showing that a 5,086-bp *Eco*RI fragment is expected from the control AF293.1 strain, whereas an 8,785-bp fragment, containing the additional 3,699 bp of the inserted transposon, is expected for transformants 1–6. **B** Blot probed with a 1,206-bp fragment of *A. fumigatus argB*. **C** Blot probed with a 2,000-bp *Xba*I-excised fragment of *pyr4*

randomly insert single simple insertions into target DNA (Biery et al. 2000). In vitro transposition was recently used to initiate genome-wide mutagenesis studies in filamentous fungi (Hamer et al. 2001). The transposon-based insertional strategy circumvents the need to identify suitable restriction sites inside the target gene into which a selectable marker can be inserted. It takes advantage of the availability of the recently sequenced *A. fumigatus* genome to PCR-amplify the gene of interest and its flanking sequences directly from *A. fumigatus* genomic DNA. The entire procedure, from DNA amplification to generation of the desired mutant strain can be performed in as little as 3–4 weeks. The incorporation of dominant selectable markers such as the hygromycin resistance cassette into the GPS-1 transprimer could make this system useful for additional fungal species in which auxotrophic strains are lacking.

To reduce copying error and increase the yield, we used the Expand high fidelity PCR system (Roche), which displays a greatly reduced error rate (8.5×10^{-6}) as compared with conventional Taq polymerase and is widely used for a variety of cloning needs. The primers used for this purpose contain restriction sites for *Asc*I, an enzyme recognizing a rare 8-bp palindromic sequence not found within the amplified DNA. Following cloning of the amplified DNA fragment into a T/A cloning vector, random insertional mutagenesis is performed using a derivative of the GPS-1 transprimer 1 transposon containing a copy of the *N. crassa pyr4* gene. Plasmids containing an insertion in the target gene are identified and mapped by PCR. In this way, plasmids containing insertions in different parts of the target gene can be rapidly isolated and used for transformation and gene-mapping. Because the steps involved in the construction of the knockout vector and its transformation into *A. fumigatus* are standardized and therefore amenable to automation, this method is particularly useful for the parallel preparation of insertional mutants for multiple genes. We used the gene-insertion strategy outlined above to obtain a mutant of *A. fumigatus* deficient in *argB*. The *A. fumigatus argB* gene was chosen for several reasons:

A. *argB* is frequently used as a selectable marker for the transformation of *Aspergillus* species such as *A. niger* (Lenouvel et al. 2002) and, based on the results presented here, it could also be used for *A. fumigatus*.
B. *argB* is an auxotrophic marker which is sometimes preferable over one derived through antibiotic resistance.
C. Mutation in *argB* of *A. fumigatus* has not yet been characterized and only a single auxotrophic marker (*pyrG*) currently exists for this organism.

In summary, we used a novel in vitro transposon/insertional mutagenesis approach to clone and disrupt the *A. fumigatus argB* gene. The resulting auxotrophic strains were dependent on externally added arginine for growth and could be transformed to arginine prototrophy using a vector containing the *A. fumigatus argB* gene. Both the mutagenesis approach and arginine auxotroph *A. fumigatus* strains described in this study

could be of value as tools for the molecular manipulation of this pathogenic fungus.

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