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Isogenic auxotrophic mutant strains in the *Aspergillus fumigatus* genome reference strain AF293

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Abstract *Aspergillus fumigatus* is a ubiquitous fungus that is a frequent opportunistic pathogen in immunosuppressed patients. Because of its role as a pathogen, it is of considerable experimental interest. A set of auxotrophic isogenic strains in the *A. fumigatus* genome reference strain AF293 has been developed. Using molecular genetic methods, arginine and lysine auxotrophs were made by deletion of *argB* and *lysB*, respectively. Transformation of these auxotrophic strains with plasmids carrying *argB* or *lysB*, respectively, results in efficient integration at these loci. Finally, these strains are able to form stable diploids, which should further facilitate analysis of gene functions in this fungus. Furthermore, the development of this isogenic set of auxotrophic strains in the AF293 background will enable investigators to study this important opportunistic human pathogen with greater facility.

Keywords Auxotroph · Transformation · Homologous recombination

Introduction

Saccharomyces cerevisiae, *Scizhosaccharomyces pombe*, *Neurospora crassa* and *Aspergillus nidulans* are all organisms with well-studied classical genetic systems

(Fincham 1989; Grallert et al. 1993; Grimm et al. 1988; Ma et al. 1987; Miller et al. 1985; Prado and Aguilera 1994; Upshall 1986; Wright et al. 1986). Because many researchers have worked on these fungi, a number of discoveries of biological importance have been made using these organisms. In addition, there is a wealth of information on their metabolic pathways and they were the first eukaryotic systems for which DNA-mediated transformation systems were applied. The filamentous fungal organisms became the paradigms for the development of transformation systems in a number of other fungal species whose genetics were less well-understood. Among the latter are the medically important fungi *Candida albicans*, *Cryptococcus neoformans*, *Histoplasma capsulatum*, *Coccidioides immitis* and *Aspergillus fumigatus*, as well as many other plant pathogenic species (Carrol et al. 1994; Fincham 1989; Fonzi and Irwin 1993; Kwon-Chung et al. 1998; Magrini and Goldman 2001; McDade and Cox 2001; Nelson et al. 2003; Reichard et al. 2000). Despite the development of transformation systems for these fungi, sophisticated molecular tools for their study are still lacking for many of them. For example, in *A. fumigatus* homologous recombination is generally thought to be inefficient and there are no isogenic strains that have different auxotrophic mutations (De Lucas et al. 2001; d'Enfert 1996; Firon et al. 2002, 2003; Kwon-Chung et al. 1998; Langfelder et al. 2002; Weidner et al. 1998).

The presence of a reference genome for the human opportunistic pathogenic fungus *A. fumigatus* facilitates molecular genetic manipulation of this fungus. However, molecular manipulations of the genome would be even easier if a number of isogenic auxotrophic mutant strains with multiple markers made in the reference strain were available. We report here the development of an isogenic strain set made in the sequence reference strain AF293. We further demonstrate that homologous recombination is efficient in these strains and, as reported for other strains of *A. fumigatus*, they are capable of forming stable diploids (Firon et al. 2002, 2003). The set of isogenic strains provides an important tool that

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will allow studies of gene function in *A. fumigatus*, much as it has done for other fungal pathogens of humans (Fonzi and Irwin 1993; Kwon-Chung et al. 1998; Magrini and Goldman 2001).

Materials and methods

Aspergillus nidulans and *A. fumigatus* strains, and growth conditions

The *A. nidulans* and *A. fumigatus* strains used in these studies and their genotypes are described in Table 1. The fungi were cultured in minimal medium (MM, 70 mM NaNO₃, 7 mM KCl, 4 mM MgSO₄, 12 mM KPO₄ pH 6.8, trace elements, 1% glucose) (Cove 1966) containing the appropriate nutritional supplements to support optimal growth (Table 1). *A. fumigatus* strain AF293 was routinely cultured at 37°C on MM. Malt extract medium was used for some experiments and consisted of 2% malt extract, 0.2% peptone, 1% dextrose, trace elements and supplements added for optimal growth as indicated in Table 1. This medium was supplemented with uridine and uracil (Table 1) for *pyrG* mutants and also contained 5F- orotic acid at 1 g/l for counterselection of the *pyr*-4-blaster, described in detail below. Agar was added at 20 g/l for solid media.

Construction of *A. fumigatus* genomic DNA library

Genomic DNA was prepared from strain AF293 as previously described (Oshero et al. 2000). Aliquots of 5 µg of genomic DNA were partially digested with the restriction endonuclease *Sau*3AI. Following digestion, the DNA was size-fractionated on a 0.7% agarose gel. A DNA fraction of 4–9 kb was cut from the gel and recovered by electroelution onto dialysis membrane in 1×TAE buffer (Sambrook et al. 1989). The DNA was recovered from the dialysis membrane, concentrated by ethanol precipitation and suspended in water at 100–200 ng/µl. The genomic DNA was ligated to the autonomously replicating vector pRG3-AMA1-*Not*I, which had been digested with *Bam*HI and dephospho-

rylated with calf intestinal alkaline phosphatase, as previously described (Oshero et al. 2000; Oshero et al. May 2000). The library contained approximately 20,000 recombinant clones, with an average insert of about 6 kb, and should thus be representative of clonable regions of the *A. fumigatus* genome.

A. nidulans transformation, plasmid rescue, and other molecular methods

The *A. fumigatus* genomic library was used to transform *A. nidulans* *argB*, *luA*, *lysB* and *riboB* auxotrophic mutants (Table 1) to prototrophy using previously described transformation conditions (Oshero et al. 2000; Oshero et al. May 2000). Transformants were selected on the appropriately supplemented MM plates made with 0.2 M sucrose and plated in 3 ml top agar with 1 M sucrose as an osmotic stabilizer. Prototrophic colonies were identified after 48–72 h of growth at 37°C.

Genomic DNA was prepared from four to six prototrophic transformant colonies for each marker after colony expansion on a nutritionally selective growth medium, as described previously (Oshero et al. 2000). Competent *Escherichia coli* (XL10-Gold, Stratagene, La Jolla, Calif., USA) were transformed with genomic DNA to recover the complementing plasmid. Plasmid DNA was prepared and digested with the restriction endonuclease *Eco*RI and fragments were separated on 0.7% agarose 1×TAE gels (Sambrook et al. 1989). Restriction digestion fragment data indicated that the plasmids recovered from independent transformant strains overlapped substantially and in some cases were identical. The appropriate auxotrophic *A. nidulans* strain was transformed with the plasmid, and from those shown to produce prototrophic transformants at high efficiency one was selected for further analysis. The complementing gene was identified by transposon insertional inactivation using the GPS system (New England Biolabs, Beverly, Mass., USA), as previously described (Oshero et al. 2000; Oshero et al. May 2000). Using this complementation of *A. nidulans* mutants, the *A. fumigatus* genes *AfuargB*, *AfuluA*, *AfulysB* and *Afu-riboB* were cloned.

Table 1 *Aspergillus* strains used in this study

Strain	Genotype	Medium supplement
<i>A. nidulans</i> A406 ^a	<i>luA1 yA2</i>	0.5 g leucine/l
<i>A. nidulans</i> A612	<i>AcrA1; riboB2 chaA1</i>	2 mg riboflavin/l
<i>A. nidulans</i> 20.1.8	<i>argB2</i>	0.5 g arginine/l
<i>A. nidulans</i> 17.1.16	<i>pyrG89 pabaA1; lysB5</i>	0.5 g lysine/l, 1.2 g uridine/l, 0.56 g uracil/l, 1 mg <i>p</i> -aminobenzoic acid/l
<i>A. fumigatus</i> AF293	Wild-type	
<i>A. fumigatus</i> AF293.1 ^b	<i>pyrG1</i>	1.2 g uridine/l, 0.56 g uracil/l
<i>A. fumigatus</i> AF293.6 ^c	<i>argB1, pyrG1</i>	0.5 g arginine/l, 1.2 g uridine/l, 0.56 g uracil/l
<i>A. fumigatus</i> AF293.7 ^c	<i>lysB1, pyrG1</i>	0.5 g lysine/l, 1.2 g uridine/l, 0.56 g uracil/l

^aStrains designated A are from the Fungal Genetics Stock Center at the University of Missouri, Kansas City, Mo. USA

^bThis strain was from our own collection and previously described (Oshero et al. 2001)

^cThese strains were developed during this study

Construction of *A. fumigatus* transformation plasmids

AfuargB was cloned as a 2,536 bp *EcoRI*–*HindIII* fragment into Bluescript (Stratagene) to generate the plasmid pargB2. *AfulysB* was cloned as a 3,370 bp *XbaI*–*EcoRI* fragment in which the *EcoRI* site was derived from the pRG3-AMA-*NotI* vector and included the *SmaI*, *KpnI* and *SacI* restrictions sites from the polylinker of that vector, resulting in the plasmid plysB3. A recyclable transformation system that is a modification of the *pyrG*-blaster method, previously described (d'Enfert 1996) was constructed. Briefly, *pyr-4* from *N. crassa* was obtained from the plasmid pODC (May et al. 1985) as a *SmaI*–*PvuII* fragment that had *BglII* linkers added and was cloned into a modified Bluescript plasmid that had a *BglII* site created at the *EcoRV* site by linker ligation. *pyr-4* was cloned between 956-bp direct repeat sequences of the kanamycin resistance gene from the vector pT-Adv (Clontech Laboratories, Palo Alto, Calif., USA). The kanamycin resistance gene sequences were amplified using PCR with the primers Neo 5' *XbaI* and Neo 3' *BamHI* for one repeat and Neo 5' *BamHI* and Neo 3' *EcoRI* for the other. The sequences of these and all oligonucleotides used in this study are provided in Table 2. The PCR products were digested with the appropriate restriction endonucleases, as indicated by their names and ligated together with Bluescript digested with *XbaI* and *EcoRI*. The resulting plasmid containing direct repeats of the kanamycin resistance gene sequences was digested with *BamHI* and dephosphorylated with calf intestinal alkaline phosphatase. *pyr-4* flanked by *BglII* sites was ligated to this to generate the *pyr-4*-blaster cassette.

The 5' and 3' DNA sequences flanking *AfuargB* were amplified by PCR using the primers argB5'-1, argB5'-2 and argB3'-1, argB3'-2, respectively. Each PCR product was approximately 1.5-kb long and introduced restriction endonuclease sites that allowed their directional cloning around the *pyr-4*-blaster cassette. Similarly, approximately 1.5 kb of 5' and 3' DNA sequences flanking *AfulysB* were PCR amplified using the primers lysB5'-1, lysB5'-2 and lysB3'-1, lysB3'-2, respectively, and cloned into the *pyr-4*-blaster

vector using the restriction endonuclease sites introduced during DNA amplification. For both genes, the oligonucleotide pairs were designed to begin at the first base before the initiator methionine or after the stop codon of the predicted polypeptides, thus leading to a complete loss of the polypeptide-coding region of the gene following integration by homologous recombination. AF293.1 protoplasts were transformed with these two plasmids after being made linear by digestion with *NotI*, and *argB* and *lysB* auxotrophs were identified among the transformant colonies. The expected gene replacement was confirmed by Southern blot analysis. To evict *pyr-4* from the strain, conidia were streaked onto malt extract medium with 5F- orotic acid as described above. Eviction of the plasmid was confirmed by Southern blot analysis and resulted in replacement of *argB* and *lysB* coding sequences by 956 bp of kanamycin resistance gene sequences.

Construction of diploid *A. fumigatus* strains

Diploid *A. fumigatus* strains were made by inoculating spores of strains AF293.6 and AF293.7 into culture tubes (13×100 mm) with 2 ml of solid malt extract medium supplemented with uridine, uracil, arginine and lysine overlaid with 2 ml of sterile water. The hyphal mat that formed on the surface of the water after 48 h at 37°C was transferred to a culture dish containing sterile water, and tweezed apart into small fragments that were plated onto MM plus uridine and uracil (MMUU). After 3 days incubation at 37°C, fans of heterokaryotic growth emerged from the pieces of hyphal mat and tips of the heterokaryotic growth cut from the agar surface were transferred to fresh plates and incubation continued. Conidia were then harvested from the surface of the heterokaryon after 48 h of growth and dilutions of these were plated on MMUU to allow the formation of colonies from single spores. Putative diploid colonies were identified as colonies with a uniform growing edge. Conidia from several putative diploids grew independently of arginine or lysine in the growth medium but still required uridine and uracil for growth.

Table 2 Sequences of oligonucleotides used in these studies

Oligonucleotide name	Sequence
Neo 5' <i>XbaI</i>	GCTCTAGATCTGGTAAGGTTGGGAAGCCC
Neo 5' <i>BamHI</i>	CGGGATCCTCTGGTAAGGTTGGGAAGCCC
Neo 3' <i>BamHI</i>	CGGGATCCAAAGGGAATAAGGGCGACACG
Neo 3' <i>EcoRI</i>	CGGAATTCAAAGGGAATAAGGGCGACACG
argB5'-1	GCGCTCTAGATGGATGAGGTTGAAATATGGA
argB5'-2	AAGGAAGCGGCCGCGTCAAACGCCAATGCGCCACT
argB3'-1	GCGCGAATTCAAATCGAATGGTTTCTATAGA
argB3'-2	GCGCGGTACCTTCGACACCGAATAGTGTC
lysB5'-1	AAGGAAGCGGCCGCGACGCTCGGTGGCGTCAATGGTG
lysB5'-2	GCGCTCTAGATGTGAGCTTCGGGTGTTGTGT
lysB3'-1	GCGCGAATTCATGATGGAGGCTGCGATGGGA
lysB3'-2	GCGCGGTACCTAGTAGATGTGGTGGTAGCAT

Results

Characterization of *argB* and *lysB* and predicted polypeptides

A. fumigatus argB and *lysB* were cloned by complementation of the corresponding *A. nidulans* mutants using an *A. fumigatus* genomic library constructed in the autonomously replicating vector pRG3-AMA1-NotI. The complementing gene was identified using transposon-mediated insertional mutagenesis and DNA sequencing. The predicted ARGB protein of *A. fumigatus* was 371 amino acids compared to 359 for that of *A. nidulans*, each encoding an ornithine carbamoyltransferase. The larger predicted protein of *A. fumigatus* was accounted for by an amino-terminal extension. The predicted LYSB protein of *A. fumigatus* was 358 amino acids and that of *A. nidulans* was 360 amino acids long (accession number AY646885). Each of the predicted polypeptides coded for a homo-isocitrate dehydrogenase, most similar to Lys12p of *S. cerevisiae*. The two predicted LYSB proteins differed at only 34 amino acid positions, with 16 of those differences being conservative substitutions. The coding region of both genes was interrupted by three introns that were at the same positions in both species and were confirmed by sequencing of cDNA clones from *A. fumigatus*.

Construction of *argB* and *lysB* auxotrophs in strain AF293

A variation of the *pyrG*-blaster method was used to generate *argB* and *lysB* deletion mutants in the *pyrG1* mutant strain AF293.1. Both the *argB* and *lysB* two-step gene replacement vectors would replace the entire predicted polypeptide-coding region of each gene on homologous integration in the first step. In the second step, following counter selection on 5F-orotic-acid-containing medium and loss of *pyr-4*, a single copy of the kanamycin resistance gene DNA sequences remains in place of the coding region of each gene. (Figs. 2, 3).

Construction of diploid strains

Diploids are useful in molecular genetic studies of gene functions, as heterozygous deletions of essential genes can be maintained. In addition, they provide the opportunity to use parasexual genetics in strain development. Methods developed for making diploids in *A. nidulans* were employed to make diploids in *A. fumigatus*. Strains with different nutritional markers were initially grown on medium that would support the growth of both strains. The hyphal mat that formed was broken into fragments and placed on plates containing no nutritional supplementation for the two forcing markers, which in this case were arginine and lysine. Pre-

sumptive diploid colonies were then identified after plating dilutions of conidia isolated from the heterokaryon that formed colonies with a uniform margin and grew independently of the presence of arginine or lysine in the medium. The isolated colonies were shown to be diploids by Southern blot analysis of genomic DNA because they were heterozygous at both the *argB* and *lysB* loci (Fig. 1).

Transformation of *argB* (AF293.6) and *lysB* (AF293.7) mutant strains with circular and linear plasmid DNA

The ability to transform each of the haploid AF293.6 or AF293.7 strains constructed was tested using the homologous gene as either circular plasmid DNA or plasmid DNA that had been linearized with *EcoRI*. Homologous recombination at *argB* and *lysB* was found to be efficient (Figs. 2, 3; Table 3). Circular plasmid

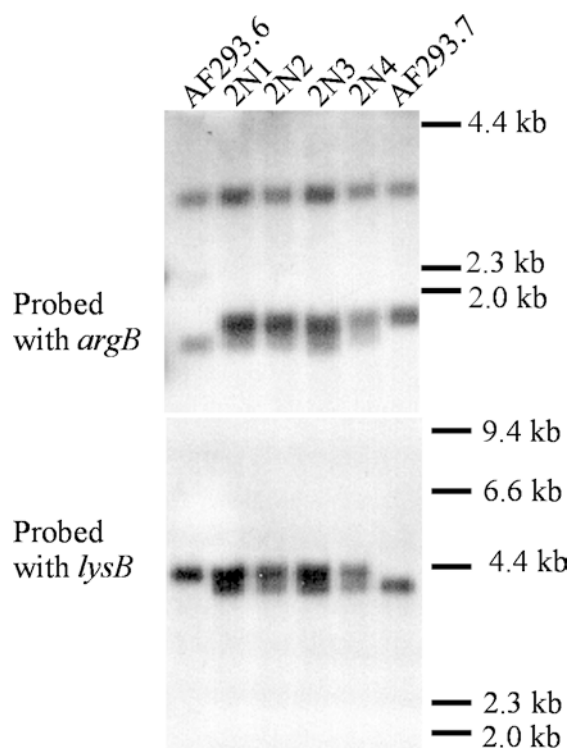


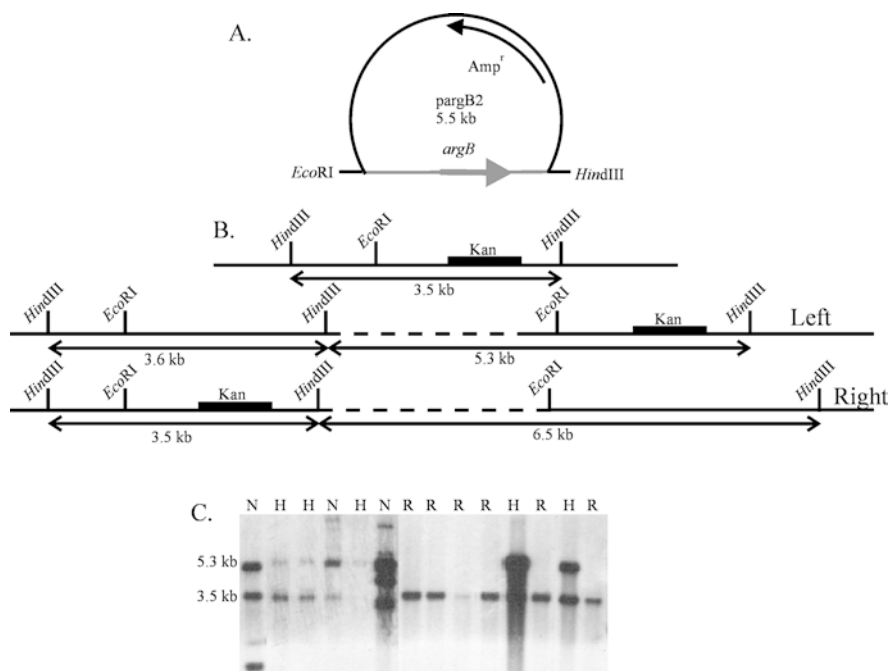
Fig. 1 Southern blot of AF293.6 (*argB1*, *pyrG1* mutant strain) and AF293.7 (*lysB1*, *pyrG1* mutant strain) parental haploid strains and four diploid strains heterozygous for *argB* deletion and *lysB* deletion, 2N1, 2N2, 2N3 and 2N4. Genomic DNA was digested with *SacI* and run on a 0.8% agarose gel, blotted, and probed with *argB* (top panel) or *lysB* gene (bottom panel). Note that the lower band in the AF293.6 strain is 150 bp smaller than in the AF293.7 strain and that both bands are present in the diploid strains. The faster migrating band of hybridization in the AF293.6 strain and diploids is weaker because the *argB* sequences have been replaced with Kan sequences. The upper band in each lane is expected and should not be altered in migration by deletion of *argB*. Only one band of hybridization is expected for *lysB*, and the migration of the band in the AF293.7 strain should be ~0.4 kb smaller. Both bands are seen in the diploid strains, as would be expected

DNA integrated efficiently at the homologous site in the chromosome, 79% of the time for *argB* and 100% of the time for *lysB*. In contrast, linear plasmid DNA integrated at lower efficiency, 44–85%. Furthermore, gene replacements occurred at these two loci with both circular and linear plasmid DNA (Table 3). Interestingly, *pargB2* and *plyB3* have limited regions of DNA homology with the chromosomal locus because the polypeptide-coding regions of the two genes have been replaced by kanamycin resistance gene sequences. The regions of homologous DNA are limited to 972 bp and 442 bp at the 5' and 3' flanking region of the gene, respectively, for *AfuargB*. Similarly, there are 1,211 bp and 874 bp at the 5' and 3' flanking region of the gene, respectively, for *AfulysB*. This suggests that even relatively short stretches of homology may provide for efficient recombination.

Discussion

Auxotrophic mutant strains and plasmids have been developed that will facilitate functional analysis of genes in the human pathogenic fungus *A. fumigatus*. Genes were cloned from *A. fumigatus* by functional complementation of the corresponding *A. nidulans* mutants. Using this approach, the *A. fumigatus* genes *AfuargB*, *AfulysB*, *AfuluA* and *AfuriboB* were cloned. For two of these genes, *AfuargB* and *AfulysB*, a well-defined forward and reverse selection system was used to make auxotrophic mutants (d'Enfert 1996). Homologous recombination was found to be efficient in the *A. fumigatus* AF293 derived strains using the corresponding plasmids. Circular plasmids integrated by homologous recombination at frequencies of greater than 80%, whereas linear molecules resulted in gene replacements by homologous recombination at frequencies of 44–85%. These frequencies are similar to those previously reported for *A. fumigatus* (Calera et al. 1997; Mellado et al. 1996a,b). We do not fully understand why this degree of variation for linear transforming DNA was obtained, but one possibility is that chromatin in the different loci was in different conformational states and thus influenced the homologous recombination by linear DNA. Additional experiments will be required to resolve this issue. The efficiency of homologous recombination in the AF293 strain should facilitate future gene-function studies in this genetic background. Our experiments also suggest that even shorter segments of DNA flanking the selective markers may be used since reasonable rates of homologous recombination at the *AfuargB* and *AfulysB* loci, with less than 1,000 bp of homology, were observed.

Fig. 2 **a** Structure of plasmid *pargB2*. The black line indicates vector sequences and the gray line represents the genomic *Aspergillus fumigatus* sequences. The arrows indicate the location and direction of transcription for *argB*, and the ampicillin resistance genes on the plasmid. **b** Structure of the *argB* locus where kanamycin resistance gene sequences have replaced *argB* coding sequences, and the two possible integration events at this site in the chromosome. Note that *pargB2* has 972 bp of homologous DNA sequences 5' and 442 bp 3' of the kanamycin DNA sequences, respectively, for homologous recombination. **c** Southern blot of genomic DNA from AF293.6 transformed with circular *pargB2*. Genomic DNA was digested with *HindIII*, separated by agarose gel electrophoresis and transferred to a nylon membrane. The blot was probed with *pargB2*. H indicates homologous recombination, N nonhomologous recombination and R is replacement, which is counted as homologous recombination in Table 3



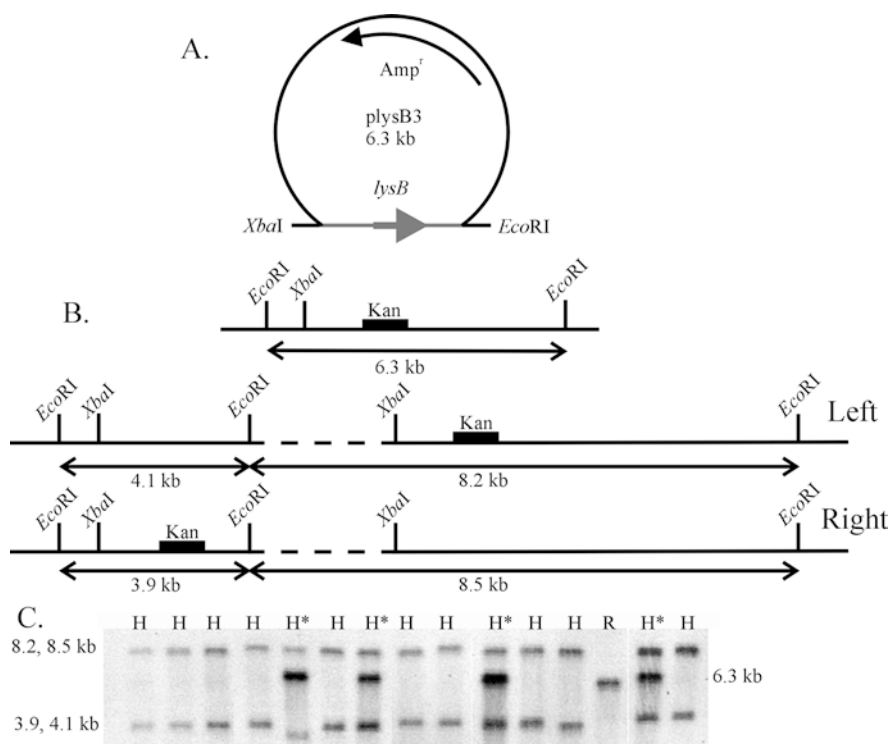


Fig. 3 **a** Structure of plasmid plysB3. The black line indicates vector sequences and the gray line represents the genomic *A. fumigatus* sequences. The arrows indicate the location and direction of transcription for *lysB*, and the ampicillin resistance genes on the plasmid. **b** Structure of the *lysB* locus where kanamycin resistance gene sequences have replaced the *lysB* coding sequences, and the two possible integration events at this site in the chromosome. Note that plysB3 has 1,211 bp of homologous DNA sequences 5' and 874 bp 3' of the kanamycin DNA sequences, respectively, for homologous recombination. **c** Southern blot of genomic DNA from AF293.7 transformed with circular plysB3. Genomic DNA was digested with *EcoRI*, separated by agarose gel electrophoresis and transferred to a nylon membrane. The blot was probed with plysB3. H indicates homologous recombination, H* is for homologous recombination with a tandem integration of the plasmid, and R means replacement, which is counted as homologous recombination in Table 3

The stable diploids obtained with AF293.6 and AF293.7 auxotrophic mutant strains should have a number of applications. For example, diploids can be used to establish a genetic map to link segments of the physical contigs from genome sequencing or to derive new combinations of auxotrophic mutations through parasexual genetics. Furthermore, diploids are useful in

analysis of gene functions and to demonstrate whether a gene is essential (Firon et al. 2002, 2003).

How nutritional auxotrophies impact virulence is an important question that needs to be addressed. It is likely that both the *argB1* and *lysB1* mutant strains will have reduced virulence or are even nonpathogenic. Reduced virulence or nonpathogenicity has been reported for *pyrG*, *pabaA*, and *areA* mutant strains of *A. fumigatus*, and *lysA2* and *pabaA1* mutant strains of *A. nidulans* (Brown et al. 2000; d'Enfert et al. 1996; Hensel et al. 1998; Tang et al. 1994). In the fungal pathogen *C. albicans*, the site of integration of the complementing nutritional marker, in addition to the auxotrophy, has been shown to impact virulence, and only recently some of the more subtle effects have been fully appreciated (Staab and Sundstrom 2003). Thus, it is well established that nutritional auxotrophies can impact virulence, which highlights the importance of caution in the use of such strains for virulence studies.

The strains described in this study along with the reasonable frequency of homologous recombination in this system now make it feasible to develop a gene

Table 3 Summary of recombination data for circular and linear DNA constructs transformed

Plasmid and form	Homologous	Non-homologous	Total	Frequency (%)
pargB2 circular	11	3	14	79
pargB2 linear ^a	23 ^b	4	27	85
plysB3 circular	15	0	15	100
plysB3 linear	7	9	16	44

^aPlasmid DNA was made linear by digestion with *EcoRI* for both pargB2 and plysB3

^bFive of these transformant strains replaced the kanamycin gene sequences resulting a gene replacement but also had additional bands indicating that other integration events had occurred

knockout set of strains for this fungus. This could be done in either haploid or diploid strains, facilitating the isolation of disruptions for essential as well as nonessential genes. A collection of gene disruption mutants in *A. fumigatus* will facilitate studies of the role of individual genes in pathogenesis and virulence in animal models. This collection may also help in the identification of novel antifungal agents as some viable mutants may display hypersensitivity to candidate compounds.

The development of an isogenic set of strains based on the reference genome strain AF293 should also facilitate molecular genetic studies in this important human pathogen. The development of similar strain set in *C. albicans* (Fonzi and Irwin 1993) resulted in their adoption as a standard strain set for studies of gene function in this pathogenic fungus. The strains we have developed will likely be similarly adopted for future studies of gene function in *A. fumigatus*. All strains and plasmids described in this report will be made available through the Fungal Genetics Stock Center (University of Missouri, Kansas City, Mo. USA).

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