Characterization of Essential Genes by Parasexual Genetics in the Human Fungal Pathogen *Aspergillus fumigatus*: Impact of Genomic Rearrangements Associated With Electroporation of DNA

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ABSTRACT

We have evaluated the usefulness of parasexual genetics in the identification of genes essential for the growth of the human fungal pathogen *Aspergillus fumigatus*. First, essentiality of the *A. fumigatus AfFKS1* gene, encoding the catalytic subunit of the β-(1,3)-glucan synthase complex, was assessed by inactivating one allele of *AfFKS1* in a diploid strain of *A. fumigatus* obtained using adequate selectable markers in spore color and nitrate utilization pathways and by performing haploidization under conditions that select for the occurrence of the disrupted allele. Haploid progeny could not be obtained, demonstrating that *AfFKS1* and, hence, β-(1,3)-glucan synthesis are essential in *A. fumigatus*. Second, random heterozygous insertional mutants were generated by electroporation of diploid conidia with a heterologous plasmid. A total of 4.5% of the transformants failed to produce haploid progeny on selective medium. Genomic analysis of these heterozygous diploids led in particular to the identification of an essential *A. fumigatus* gene encoding an SMC-like protein resembling one in *Schizosacccharomyces pombe* involved in chromosome condensation and cohesion. However, significant plasmid and genomic DNA rearrangements were observed at many of the identified genomic loci where plasmid integration had occurred, thus suggesting that the use of electroporation to build libraries of *A. fumigatus* insertional mutants has relatively limited value and cannot be used in an exhaustive search of essential genes.

ASPERGILLUS fumigatus is a ubiquitous filamentous fungus of human environments and an opportunist pathogen (for review see Latgé 1999, 2001). Dissemination of *A. fumigatus* occurs by release of asexual spores, called conidia, into the atmosphere. Due to their small diameter (2–3 μm), conidia can reach the lung alveolae and cause invasive pulmonary aspergillosis (IPA) when innate immunity is deficient. With the rising number of immunocompromised hosts and the development of severe immunosuppressive therapies, the incidence of IPA has dramatically increased to become the most frequent airborne fungal infection in developed countries (McNeil *et al.* 2001). Because of a difficult diagnosis and the lack of efficient, nontoxic antifungal treatments, IPA is associated with a mortality rate as high as 85% (Lin *et al.* 2001).

A rational approach to develop new therapies relies on the identification of virulence factors (Perfect 1996). Despite intensive efforts, no genuine virulence factor has been identified yet in *A. fumigatus* (reviewed in d'Enfert 2000; Latgé 2001). This suggests that *A. fumigatus* virulence is a multifactorial process that depends mostly on the host immune system status, reflecting the opportunistic pathogenicity of this species (Odds *et al.* 2001). An alternative strategy for finding novel antifungal targets might be in the identification of genes that are essential for fungal growth under *in vitro* growth conditions (Reich 2000). Compendia of essential genes have been obtained for the yeast *Saccharomyces cerevisiae* through various approaches including identification and characterization of temperature-sensitive mutants, systematic gene inactivation, or insertional mutagenesis in a diploid background followed by the analysis of meiotic progeny (Ross-Macdonald *et al.* 1999; Winzeler *et al.* 1999). More recently, a compendium of *Candida albicans* essential genes has been produced by expressing antisense RNA molecules (De Backer *et al.* 2001).

Several of these approaches are not yet applicable to filamentous fungi, mainly because of the lack of complete genome sequences and of the low efficiency of homologous recombination (d'Enfert *et al.* 1999). In this regard, insertional mutagenesis in a diploid back-

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ground appears as the most suitable method to identify essential genes in filamentous fungus. Aspergillus species are natural haploids that reproduce asexually, although a sexual cycle is performed by some species, in particular *A. nidulans*, when diploids are transiently formed. Alternatively, some fungi undergo a parasexual cycle, as first described by Pontecorvo *et al.* (1953) in *A. nidulans* and subsequently in other fungal species including *A. fumigatus* (Stromnaes and Garber 1963). Stable diploids can be obtained using adequate genetic markers in the parent strains and appropriate selection procedures. These diploids do not enter meiosis but destabilizing reagents like benomyl or *p*-fluorophenylalanine can be used to induce the formation of haploid segregants because of mitotic chromosomal nondisjunction (Hastie 1970; Timberlake and Marshall 1988; Timberlake 1991; Clutterbuck 1992).

Parasexual genetics has been used to demonstrate gene essentiality in *A. nidulans* (Timberlake 1991; Som and Kolaparthi 1994). In these experiments, one allele of the target gene is inactivated by homologous gene replacement in a stable diploid. The absence of the inactivated allele in all haploid segregants indicates that the gene is essential for fungal growth. This strategy has not yet been used to identify essential fungal genes without prior sequence information by random insertional mutagenesis of a stable diploid strain.

Currently available procedures for random insertional mutagenesis in *A. fumigatus* rely on the integration of plasmid DNA into the fungal genome using transformation. Work by Weidner *et al.* (1998) and Brown *et al.* (1998) has shown that electroporation of intact conidia with a nonhomologous plasmid is more appropriate than protoplast transformation for the generation of collections of insertional mutants since it provides (1) high transformation efficiencies, (2) integration of only one copy of the transforming DNA in the majority of transformants, and (3) an apparent random distribution of the plasmid in the genome, as judged from the various sizes of restriction fragments that have integrated the transforming DNA molecule. Addition of restriction enzymes during protoplast transformation (restriction enzyme-mediated integration) also appears to increase transformation efficiency and to favor single-site insertion of the transforming DNA (Brown *et al.* 1998). Although electroporation has been applied to generate a large collection of insertional mutants in a haploid background and subsequent screening of the mutants in an animal model of IPA, little is known about the consequence of plasmid integration on the genome structure in *A. fumigatus* (Brown *et al.* 2000).

Here, we have combined parasexual genetics and insertional mutagenesis for the systematic identification of essential genes in *A. fumigatus* at a genomic scale. First, we have validated this strategy by inactivating one allele of a presumed essential *A. fumigatus* gene by homologous recombination. β-(1,3)-Glucan is a major component of the fungal cell wall synthesized by an enzymatic complex whose catalytic subunit is encoded by the *A. fumigatus AfFKS1* gene (Beauvais *et al.* 2001). Glucan synthase is the target of the new antifungal drugs, candins (Onishi *et al.* 2000; Tkacz and DiDomenico 2001). In several species where it has been attempted (*S. cerevisiae*, *Cryptococcus neoformans*), inactivation of the homologous gene is lethal (Mazur *et al.* 1995; Thompson *et al.* 1999) and repeated attempts to inactivate *AfFKS1* in a haploid strain of *A. fumigatus* have failed (Beauvais *et al.* 2001). Here, we use parasexual analysis of heterozygous diploid transformants to demonstrate that the β-(1,3)-glucan synthase enzyme is essential for the growth of *A. fumigatus*. We also generated and analyzed random insertions in diploid mutants of *A. fumigatus*. Our results show that, although the strategy has the potential to identify *A. fumigatus* genes that are essential for growth, insertional mutagenesis based on the electroporation of DNA is associated with numerous genomic rearrangements that limit the value of this approach.

MATERIALS AND METHODS

***A. fumigatus* strains and culture conditions:** *A. fumigatus* strains used in this study are listed in Table 1. *A. fumigatus* strains were propagated at 37° on complete medium or minimal medium (MM) with 0.5 mm of one of the following nitrogen sources: sodium glutamate, ammonium tartrate, sodium nitrate, sodium nitrite, or hypoxanthine (Cove 1966). Uridine and uracil were added at a concentration of 5 mm when appropriate. Selection of *A. fumigatus* mutants unable to utilize nitrate as the sole nitrogen source was obtained by plating spores on MM containing ammonium tartrate and 100 mm sodium chlorate (Cove 1966). The nature of the mutation was assessed by growth on MM supplemented with different nitrogen sources, as previously described (Cove 1976). Selection of *A. fumigatus* mutants auxotrophic for uridine and uracil was achieved on MM containing 1 mg/ml 5-fluoroorotic acid plus uridine and uracil (d'Enfert 1996). Liquid cultures used for DNA-mediated transformation and genomic DNA preparation were grown in YG (0.5% yeast extract, 2% glucose). Haploidization of *A. fumigatus* diploid strains was conducted on selective haploidization medium [SHM; complete medium containing 1.2 μg/ml benomyl (Aldrich, Milwaukee; 10 mg/ml in DMSO)] or on nonselective haploidization medium (NSHM; selective haploidization medium plus uridine and uracil).

Plasmid and DNA manipulation: General recombinant DNA techniques and Southern-blot analyses were performed essentially according to Sambrook *et al.* (1989) and Ausubel *et al.* (1992). Transformation of calcium-manganese-treated *Escherichia coli* was as described (Hanahan *et al.* 1991). Oligonucleotides used in this study were obtained from Genset (Paris) and are listed in Table 2. pCB::Fks is a derivative of pCB1004 (Caroll *et al.* 1994), which contains a hygromycin-resistance marker for selection of fungal transformants and in which a 2.3-kb *Kpn*I internal fragment [nucleotides (nt) 1342–3672] of *AfFKS1* has been subcloned (Beauvais *et al.* 2001). pF4 was obtained by subcloning an *Xba*I fragment containing the *A. niger pyrG* gene (van Hartingsveldt *et al.* 1987) into pCB::Fks. ppyrG, a pUC18 derivative containing the *A. nidulans pyrG* gene (Oakley *et al.* 1987) that efficiently complements the *A. fumigatus pyrG1* mutation (Weidner *et al.* 1998)

was kindly provided by Claudio Scazzocchio (Université Paris-Sud, Orsay, France).

Heterozygous *A. fumigatus FKS1/fks1* strains construction: Plasmid pF4 was introduced in *A. fumigatus* strain CEA136 by transformation of protoplasts as described previously (Osmani *et al.* 1987; d'Enfert 1996). Genomic DNA of transformants was prepared according to Girardin *et al.* (1993). *Eco*RV- and *Hin*dIII-digested genomic DNA was probed with the 2.3-kb *Kpn*I fragment of pCB::Fks that had been labeled using the Rediprime labeling kit (Amersham, Arlington Heights, IL) and [α-^{32}P]dCTP. Integration of pF4 at one of the *AfFKS1* loci in the diploid strain is expected to result in the appearance of two *Eco*RV fragments of 3.6 and 8.8 kb and two *Hin*dIII fragments of 1.7 and 7.4 kb in addition to the *Eco*RV fragment of 3.8 kb and two *Hin*dIII fragments of 1.25 and 1.75 kb that are also detected in genomic DNA of a wild-type diploid. Integration of pF4 at the *AfFKS1* locus is confirmed by PCR analysis. Genomic DNA amplification with oligonucleotides fks1 and fks2 yields a 2.4-kb fragment in all diploid strains with at least one wild-type *AfFKS1* allele. In strain CEA136 and in transformants with an ectopic integration of pF4, amplification with oligonucleotides T7 and fks2 does not yield any product. In contrast, amplification of genomic DNA of transformants with pF4 integrated at the *AfFKS1* locus results in a 2.6-kb fragment corresponding to the disrupted *fks1* allele when the T7 and fks2 oligonucleotides are used.

Production and characterization of *A. fumigatus* insertional mutants: *A. fumigatus* insertional mutants were obtained by electroporation of intact conidia of strain CEA136 with *Eco*RI-digested ppyrG. On average, 0.5 μg of linearized DNA was used to transform 5×10^7 conidia prepared according to Weidner *et al.* (1998). Electroporation was carried out in a 0.2-cm electroporation cuvette (Bio-Rad, Richmond, CA) and cells were subjected to a 1-kV pulse using a Bio-Rad electroporation device (400 Ω, 25 μF). Transformants were selected on complete medium. Following purification to single colonies on complete medium, transformants were scored on SHM and NSHM.

Genomic DNA of transformants was prepared as described above. *Cla*I-digested genomic DNA was subjected to Southern analysis using ^{32}P-labeled ppyrG as a probe. Genomic DNA regions flanking ppyrG were amplified using the method adapted from Chun *et al.* (1997). First, ~100 ng of genomic DNA was amplified in 50 μl using oligonucleotides ppyr1 and PCRal1 or ppyr3 and PCRal1 (4 pmol/μl final) and the following amplification protocol: a denaturation step at 94° for 3 min followed by 5 cycles of the following steps: denaturation at 94° for 30 sec, annealing at 35° for 30 sec, extension at 72° for 1 min, and 30 cycles of the following steps: denaturation at 94° for 30 sec, annealing at 45° for 30 sec, extension at 72° for 1 min. A last elongation step was performed at 72° for 3 min. Final concentrations for $MgCl_2$ and dNTPs were 3 mm and 0.2 mm, respectively. One microliter of the PCR reaction was subjected to a second amplification using similar reaction conditions and oligonucleotides ppyr2 and PCRal2 (if ppyr1 and PCRal1 had been used in the first reaction) or ppyr4 and PCRal2 (if ppyr3 and PCRal1 had been used in the first reaction). The following amplification protocol was used: 30 cycles of the following steps: denaturation at 94° for 30 sec, annealing at 60° for 30 sec, extension at 72° for 1 min. A last elongation step was performed at 72° for 3 min. In some instances, oligonucleotides PCRal3, PCRal4, and PCRal5 were used in place of PCRal1. PCR products were separated by electrophoresis on a 2% agarose-TBE gel and major PCR products were purified with the Qiaquick gel purification kit (QIAGEN, Valencia, CA) according to the supplier's instructions. Purified PCR products were sequenced using ppyr2 or ppyr4 as primers (ESGS, Evry, France). Nucleotide sequences obtained in this manner and trimmed for ppyrG sequences were compared to protein databases and to the preliminary sequence data of the *A. fumigatus* genome project obtained from The Institute for Genomic Research (TIGR) website (6× shotgun sequencing publicly available on 14 November 2001 at http://www.tigr.org) using Blastx or Blastn (Altschul *et al.* 1990).

Genomic regions corresponding to the site of insertion of ppyrG in selected transformants were characterized as follows. Probes were prepared by PCR using appropriate primers based on the sequence obtained from the genomic DNA of transformants and labeled with [^{32}P]dCTP using the Rediprime kit. Probes were used to identify cosmids in a genomic library of *A. fumigatus* (Borgia *et al.* 1994). In the case of transformant 96, cosmid 25D9 was identified and subjected to shotgun sequencing according to standard procedures. Following assembly, the sequence of cosmid 25D9 was deposited at the GenBank nucleotide sequence database (accession no. AY080962). Oligonucleotides were designed from the sequence of cosmid 25D9 to deduce the genomic organization at the site of integration of ppyrG by PCR reactions on genomic DNA of transformant 96 and *A. fumigatus* CEA136.

RESULTS

Characterization of candidate *A. fumigatus* essential genes through parasexual genetics construction of *A. fumigatus* diploid strains: Stable *A. fumigatus pyrG* diploids heterozygous for spore color markers and for genes involved in nitrate utilization were obtained using the following strategy. Spore color mutants CEA82 and CEA85 (Table 1) of the *A. fumigatus pyrG* strain CEA17 (d'Enfert 1996) were identified by insertional mutagenesis with plasmid pAfpyrG2 (Weidner *et al.* 1998). Derivatives of these strains (CEA88 and CEA92; Table 1) that were unable to use nitrate as the sole nitrogen source were obtained by incubation in the presence of chlorate. Heterokaryons could be obtained by growing these complementary strains on MM nitrate and produced gray-green spores in addition to spores with the parental colors. Only these gray-green spores were able to use nitrate and, when incubated in the presence of benomyl, produced sectors of parental-colored spores, indicating that they correspond to *A. fumigatus* diploids. One of these diploids was designated CEA99 (Table 1). A derivative of CEA99 that had lost pAfpyrG2 sequences as indicated by Southern hybridization (data not shown) was obtained by selection on 5-fluoroorotic acid and designated CEA136 (*w1/+, +/r7, cnx1/+, +/nirA4 pyrG1/pyrG1*). Upon haploidization, strains CEA99 and CEA136 produced sectors of parental-colored spores only, suggesting that both color mutations lie on the same chromosome, as expected if these mutations affect the cluster of genes involved in the synthesis of the spore color pigment in *A. fumigatus* (Tsai *et al.* 1999).

β-(1,3)-Glucan synthase is essential for growth in *A. fumigatus*: Plasmid pF4 (Figure 1A) was used to inactivate one allele of *AfFKS1* in strain CEA136 by homologous recombination. pF4 contains an internal fragment (nt 1342–3672) of the 5813-bp *AfFKS1* open reading

TABLE 1

***A. fumigatus* strains**

Name	Genotype	Reference
CEA17	*pyrG1*	d'Enfert (1996)
CEA82	*pyrG1 w1::pAfpyrG2*	This study
CEA85	*pyrG1 r7::pAfpyrG2*	This study
CEA88	*pyrG1 w1::pAfpyrG2 cnx1*	This study
CEA92	*pyrG1 r7::pAfpyrG2 nirA4*	This study
CEA99	*pyrG1/pyrG1 w1:pAfpyrG2/+ +/r7:pAfpyrG2 cnx1/+ +/nirA4*	This study
CEA136	*pyrG1/pyrG1 w1/+ +/r7 cnx1/+ +/nirA4*	This study

w1, white mutant; *r7*, reddish mutant; *cnx*, molybdopterin synthase mutant; *nirA*, nitrate and nitrite reductase positive regulator mutant.

frame cloned into a vector carrying two fungal markers: *hph,* which confers hygromycin resistance, and *pyrG,* which confers uridine/uracil prototrophy. Analysis of nine transformants of diploid strain CEA136 revealed that two resulted from the integration of pF4 at one of the two *AfFKS1* loci (Figure 1, A and B; data not shown). Haploidization of these *AfFKS1/Affks1* heterozygous diploids was obtained on nonselective haploidization medium, which would enable the growth of haploid strains containing *AfFKS1* or *Affks1*, but not on a selective haploidization medium, which would enable the growth of only haploid strains containing *Affks1*, suggesting that inactivation of *AfFKS1* is lethal in *A. fumigatus* (Figure 1C). *AfFKS1/Affks1* heterozygous diploids form a cal on haploidization selective medium that might result from the production of poorly viable aneuploids in the presence of the mitotic spindle inhibitor benomyl, which, at low concentration, induces chromosomal nonmitotic disjunction in Aspergillus diploid strains (Hastie 1970). Essentiality of *AfFKS1* was confirmed by testing haploid conidia obtained following growth of the *AfFKS1/Affks1* heterozygous diploid on NSHM for uridine/uracil prototrophy. None of these conidia had retained the transformation marker and, hence, *Affks1* allele (data not shown). Taken together, these results suggested that *AfFKS1* is essential for growth in *A. fumigatus* and that parasexual genetics might be used to test the essentiality of a cloned *A. fumigatus*

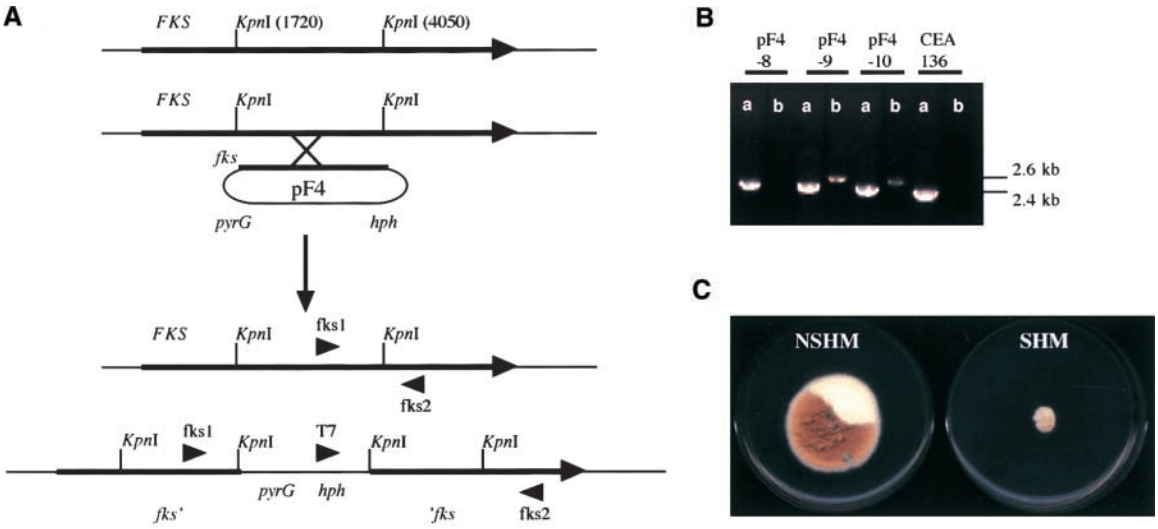

Figure 1.—Disruption of one allele of an *A. fumigatus AfFKS1* gene in a diploid strain and parasexual analysis. (A) Plasmid pF4 contains a 2.3-kb *Kpn*I internal fragment of the *A. fumigatus AfFKS1* gene and two fungal transformation markers (*pyrG* and *hph*). Transformation of diploid CEA136 protoplasts leads to the inactivation of one allele of *AfFKS1* by homologous recombination. The locations of primers fks1, fks2, and T7 used for PCR analysis of transformants are indicated by arrowheads. (B) PCR analysis of the *AfFKS1* loci in *A. fumigatus* strain CEA136 and transformants pF4-8, pF4-9, and pF4-10. The occurrence of a wild-type allele is reflected by a 2.4-kb fragment when primers fks1 and fks2 are used (a, all strains). A disrupted *Affks1* allele is visualized by the amplification of a 2.6-kb fragment when primers fks 2 and T7 are used (b, transformants pF4-9 and pF4-10). The absence of an amplification product in diploid transformants with these two primers reflects ectopic integration of pF4 (transformant pF4-8). (C) Haploidization of transformant pF4-9 on nonselective haploidization medium (NSHM) or selective haploidization medium (SHM). Inability to form haploid sectors is correlated with the formation of a cal on SHM, indicating that the *FKS1* gene is essential for the viability of *A. fumigatus.*

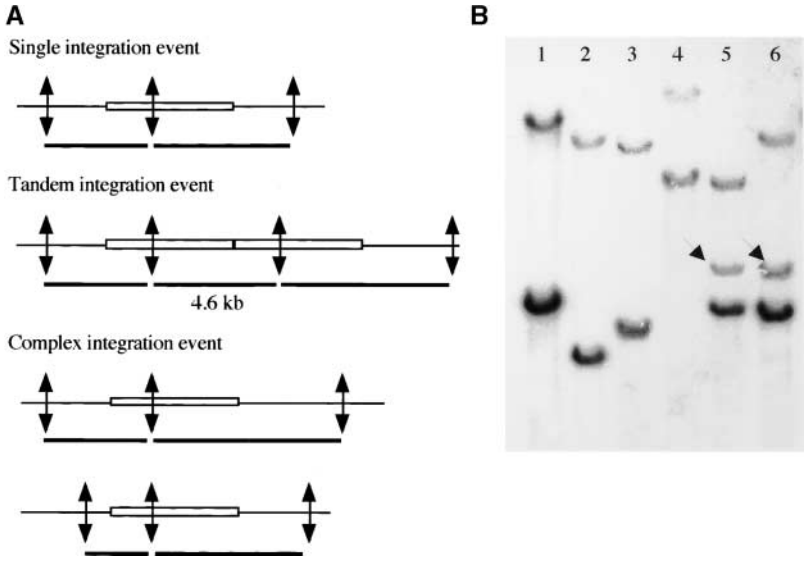

FIGURE 2.—Southern analysis of six derivatives of *A. fumigatus* strain CEA136 obtained by transformation with linearized ppyrG. (A) Schematic representation of expected integration events and hybridization data obtained using genomic DNA cleaved by a restriction enzyme (double arrow) that cuts once in the transforming DNA and the transforming plasmid (open box) as a probe. Integration of one copy of the plasmid (open box) into genomic DNA results in two restriction fragments of varying size (thick lines). Tandem integrations of the plasmid result in two hybridizing fragments of varying size plus a constant fragment of 4.6 kb. Complex integrations of the plasmid result in four or more fragments of varying size. (B) Southern analysis of six transformants of strain CEA136. Genomic DNA was digested with *Cla*I, which cleaves once in ppyrG, and probed with α-^{32}P-labeled ppyrG. Two *Cla*I fragments are observed in transformants 1–4, which have undergone a single integration event. Three *Cla*I fragments, including a 4.6-kb fragment (arrows), are observed in transformants 5 and 6, which have undergone a tandem integration event.

gene. Interestingly, the conditions that we have tested did not permit haploidization on media containing hygromycin, suggesting that *hph* is not a useful marker for parasexual genetics studies in *A. fumigatus* (data not shown).

Identification of *A. fumigatus* essential genes by insertional mutagenesis: *Random insertional mutagenesis and parasexual screening:* Transformation with a linearized heterologous plasmid (ppyrG; OAKLEY *et al.* 1987) was used to generate a collection of independent insertional mutants of strain CEA136. Electroporation of intact conidia (BROWN *et al.* 1998; WEIDNER *et al.* 1998) yielded 1000–2000 transformants per μg DNA. Southern analysis (Figure 2 and data not shown) of genomic DNA of 12 randomly chosen transformants revealed two fragments of varying sizes in transformants harboring a single copy of ppyrG, while an additional 4.6-kb fragment corresponding to ppyrG was observed when tandem integration of ppyrG had occurred, and additional fragments were observed when ppyrG has integrated at several locations. Eight transformants resulted from the integration of a single copy of ppyrG in their genome; 4 transformants had integration of tandem copies of ppyrG. Complex integration events (at least two copies of ppyrG at different genomic locations) appeared underrepresented. PCR analysis using primers ppyr1 and ppyr3 (Table 2) on 95 independent transformants confirmed that 34% (32/95) of the transformants had at least two copies of the plasmid inserted at the same genomic location. The occurrence of fragments of different sizes in the genomic DNA of transformants analyzed by Southern hybridization (Figure 2 and data not shown) is consistent with a random distribution of integration sites. However, it does not preclude the possibility of integration hotspots.

A collection of 544 independent transformants was screened for growth on SHM and NSHM. Two categories of transformants were obtained. A total of 519 (95.5%)

TABLE 2

Primer sequences

ppyr1 (5′ end)[a]	GGAAGACGGGCAGTTAGTCC
ppyr3 (3′ end)[a]	CCCAGGCTTTACACTTTATGC
PCRal1[b]	GGCCACGCGTCGACTAGTAC(N)$_{10}$GATAT
PCRal3[b]	GGCCACGCGTCGACTAGTAC(N)$_{10}$ACGTC
PCRal4[b]	GGCCACGCGTCGACTAGTAC(N)$_{10}$TGGAC
PCRal5[b]	GGCCACGCGTCGACTAGTAC(N)$_{10}$ACGTG
ppyr2 (5′ end)[a]	CGAAGTTGACGTTCAGTATGC
ppyr4 (3′ end)[a]	TGACCATGATTACGCCAAGC
PCRal2	GGCCACGCGTCGACTAGTAC
fks1	CATGATCACCAACTTCAACC
fks2	CGAGATCACGACCTTTACC
T7	TAATACGACTCACTATAGGG
96-a	ACGAAGGACGACCGTACAAC
96-b	TGATGAGCACCGTAGTGAGC
96-c	TTATTCATTGCGGAGGGAAG
96-d	ACTGGGTGGAGTTGAAGTGG
96-e	TCCCTGAATGACCAACTTCC
96-f	TCAGTGTCGAGTCGGAGTTG
96-g	ACATCCTACCATGCGGACTC
96-h	TCTCATCGTGACGAAACTCG

[a] ppyr primers are specific of the ppyrG plasmid used for insertional mutagenesis.

[b] The pentamer at the 5′ end of the random primer (PCRal1, -3, -4, and -5) is expected to bind once in 1 kb ($1/4^5$). It is chosen according to the GC% of *A. fumigatus* (~50%) and does not occur in the region of ppyrG located between ppyr primers and the linearized plasmid end.

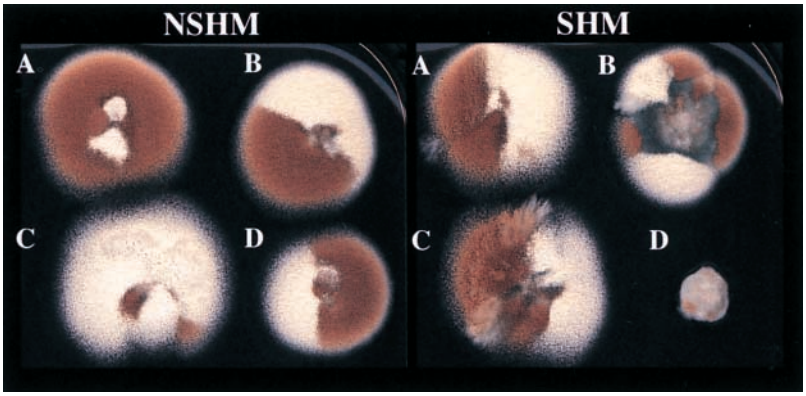

FIGURE 3.—Parasexual genetic screening to identify an essential gene. Haploidization of four diploid *pyrG*$^+$ transformants on nonselective (NSHM) and selective (SHM) haploidization media gave a cal growth phenotype on selective medium when the plasmid is integrated in an essential gene (transformant D). Random disjunction of chromosomes is visualized by the parental color of haploid conidia.

transformants produced virtually indistinguishable colonies on the two media, suggesting that integration of ppyrG in these transformants did not prevent the expression of a gene critical for the growth of *A. fumigatus* (see Figure 3 for an example, transformants A, B, and C). In contrast, 25 (4.5%) transformants produced a cal on SHM similar to the *AfFKS1/Affks1* heterozygous diploid (*e.g.*, Figure 3, transformant D). Sixteen diploid transformants in the second category were further characterized by analyzing spores produced on NSHM for their phenotypes after plating on selective and nonselective complete media. Plating 10^5 haploid spores of each yielded no haploid pyrG$^+$ colonies (data not shown). This indicated that these 16 transformants contained a copy of ppyrG genetically linked to the inability to produce haploid spores in the presence of benomyl. That is, they might have a ppyrG insertion in a gene necessary for the growth of *A. fumigatus*.

Characterization of genomic sequences at the site of plasmid integration: Since each haploid-lethal mutation should be physically marked by the insertion of a plasmid, the characterization of the junctions between the genome and the plasmid in the 16 diploid transformants described above was achieved using the method of CHUN *et al.* (1997) to identify the corresponding *A. fumigatus* genes.

Identification of the flanking genomic sequences in eight transformants was hampered because of two types of plasmid rearrangements. First, tandem plasmid integrations were observed in four transformants and confirmed by PCR analysis. Their frequency (25%) is in the range of that observed in the overall diploid transformant population (34%; see above). Second, mosaic structures with one end of the plasmid followed by internal fragments of the plasmid in different orientations and of variable length and position were observed in four transformants (data not shown).

Genomic sequences flanking plasmid DNA could be obtained for eight transformants with a single integrated copy of the plasmid (Table 3). In these, the plasmid ends have not undergone major DNA rearrangements (Table 3). In most cases (10/15), only the single-strand protruding ends (3′-TTAA-5′) resulting from *Eco*RI cleavage prior to transformation were deleted in the integrated plasmid. Two minor deletions (5 and 6 bp) were observed at one end of the plasmid in two transformants (3′ end of transformant 99 and 5′ end of transformant 503; Table 3). In some instances, nucleotides that did not originate from the plasmid or genomic DNA were observed at their junction (3′ end of transformants 96 and 516, 5′ end of transformant 197; Table 3). Comparison of genomic sequences surrounding the plasmid in the eight transformants did not reveal microhomologies between plasmid and genomic DNA, consistent with the apparent random selection of the integration sites.

Identification of a member of the structural maintenance of chromosome protein family as necessary for efficient growth in A. fumigatus: Characterization of the sequences flanking the site of integration in transformant 234 (Table 3) revealed that integration was in a gene encoding a structural maintenance of chromosome (SMC)-like protein (STRUNNIKOV and JESSBERGER 1999). The sequence of a corresponding 4339-bp genomic clone was determined (accession no. AY081008). This sequence overlaps with contig 432 (nt 6899–11240) of the *A. fumigatus* genome sequence (http://www.tigr.org/; release of November 14, 2001). The predicted polypeptide is encoded by an open reading frame (ORF) of 3561-bp nucleotides interrupted by five putative introns. The insertion of ppyrG is located at nt 996 (amino acid 332).

The deduced polypeptide sequence shows 30% identity and 50% similarity with the *Schizosaccharomyces pombe* Spr18 protein (SMC protein partner of Rad18), which is involved in the maintenance of chromosome structure and is essential for growth in this species (HIRANO 1999; FOUSTERI and LEHMANN 2000). It is also 28% identical and 48% similar to *S. cerevisiae* YOL034w, which is essential for growth (WINZELER *et al.* 1999). Alignment of the *A. fumigatus* predicted protein with members of the SMC protein family shows two blocks of conserved residues located at the amino and carboxy termini of the protein (Figure 4). These results suggest that the gene interrupted by ppyrG in transformant 234 encodes a member of the SMC protein family that is essential for growth of *A. fumigatus*.

Genomic rearrangements in transformants with a haploid-

lethal phenotype: Analysis of the seven remaining transformants (96, 99, 197, 209, 503, 516, and 536) revealed that the 5′- and 3′-flanking regions in six were homologous to different contigs of the *A. fumigatus* genome sequence (Table 3). Furthermore, amplification of genomic DNA of *A. fumigatus* strain CEA136 with primers designed on the basis of the 5′ and 3′ sequences flanking ppyrG were all unsuccessful (data not shown). For transformant 536, where only the region located in 3′ of ppyrG could be characterized, primers designed on the basis of the corresponding *A. fumigatus* contig (Contig856, http://www.tigr.org/) did not generate PCR products with primers corresponding to the 5′ end of ppyrG (data not shown). These results suggested that these seven transformants had undergone significant genomic DNA rearrangements at the site of integration of ppyrG.

This result was confirmed by the detailed characterization of transformant 96. In this case, cosmid 25D9 containing the genomic sequences flanking both ends of ppyrG was sequenced (accession no. AY080692). Comparison of the cosmid sequence with the sequence of genomic DNA flanking ppyrG in transformant 96 revealed that integration of ppyrG is actually associated with the deletion of a 16,057-bp genomic DNA fragment and the inversion of a 587-bp fragment (Figure 5). Consequently, integration of ppyrG was associated with the partial or complete deletion of at least five ORFs (Figure 5). Two of these ORFs have no significant homolog in public databases (Figure 5; 25d9-9 and 25d9-10), while three resemble *S. cerevisiae* genes, namely Mvp1p (Ekena and Stevens 1995), Nha1p (Prior *et al.* 1996), and Ste50p (Jansen *et al.* 2001). Although the three *S. cerevisiae* genes identified are not essential for yeast growth (Winzeler *et al.* 1999), our results indicate that at least one of the five genes inactivated in transformant 96 is essential for growth in *A. fumigatus.*

The comparison of the sequence of the regions flanking ppyrG in the six remaining transformants with the genomic data provided by the corresponding TIGR contig suggested that they had experienced significant genomic rearrangements (*e.g.*, deletions of at least 30.9, 20.7, and 21.7 kb at the integration sites in transformants 99, 503, and 516, respectively). Analysis of transformants 197 and 209 showed that integration had been associated with shuffling of DNA originating from various regions of the genome (Table 3). The exact nature of these rearrangements could not be deduced from the available sequence data.

Electroporation-mediated insertional mutagenesis is associated with major genomic rearrangements: To test whether the selection of transformants with an insertion of ppyrG in a gene essential for growth in *A. fumigatus* was associated with an increased frequency of chromosomal rearrangements, we analyzed the structure of the integration site in seven transformants with a single copy of ppyrG that could form haploid sectors on selective hap-

TABLE 3

Nucleotide sequence at the site of integration of ppyrG in transformants of *A. fumigatus* CEA136

Transformant	Contig length (kg)	TIGR contig (position of genomic tag)	Genomic tag (bp)	Genomic sequence (left side)[a]	Left plasmid end 5′-AATTCGAGCTCGGTA- - - - 3′-GCTCGAGCCAT- -	Right plasmid end - - - -GACAGGTATCG-3′ - - -CTGTCCATAGCTTAA-5′	Genomic sequence (right side)[a]	Genomic tag (bp)	TIGR contig (position of genomic tag)	Contig length (kb)
96	15.2	1220 (9074–9806)	735	GCGTGAAGCG	CGAGCTCGGTA- - - -	-GACAGGTATCG	CGTCCAAGCG	309	533 (11214–10909)	26.1
99	37.3	856 (14059–14458)	401	CAGAGAGGGT	ATTCGAGCTCGGTA- - - -	-GACAG	TTGTACACTG	206	407 (4434–4229)	12.5
197	11.1	nt 1 to 359: 1105 (9540–9899)	486	AACGATCCAG	TTCGAGCTCGGTA- - - -	-GACAGGTATCGA	ATTATTCTTG	244	785 (34553–34792)	75.0
	75.1	nt 357 to 483: 29 (74927–74801)								
209	14.1	nt 1 to 141: 701 (3026–3764)	180	CATGAATGGA	TCGAGCTCGGTA- -	-GACAGGTATCGAA	CCTTTCTTGC	368	152 (2290–2654)	39.9
	1.9	nt 141 to 180: 1966 (1487–1526)								
234	15.6	432 (9196–9900)	706	ATTGAAT**GAC**	TCGAGCTCGGTA- - - -	-GACAGGTATCGAATT	**GAC**TGGCCGG	403	432 (9898–10300)	15.6
503	26.5	529 (16977–16623)	352	CTATCAAAGT	TCGGTA- - - -	-GACAGGTATCGAA	AGTTGACAGC	170	565 (4128–4297)	11.8
516	14.4	818 (10371–9853)	519	TCTTCTCGAG	TTCGAGCTCGGTA- - - -	-GACAGGTATCGAATT	CATCACCCCC	193	994 (11824–12010)	18.9
536	NA	NA	NA	NA	NA	- - -GACAGGTATCGAATT	CCTAAATTGT	140	856 (7724–7862)	37.3

NA, not available.

[a] Underlined nucleotides did not originate from plasmid or target genomic DNA. In transformant 234, boldface nucleotides originate from the genomic target and are duplicated at the junctions with the integrated plasmid.

SMC_N

```
SmcA     98-GEVDDDGYKPGAIVRIKVTDFVTYTSAEFFPGPKLNMVIGPNGTGKSTLVCAICLGLGWGPQHL
Spr18     1-MILTRESYALGSIVRIKLVNFVTYDYCELFPGPYLNLIIGPNGTGKSTIVSAICIGLGWPPKLL
YOL034w  30-AKPDLSSFQPGSIIKIRLQDFVTYTLTEFNLSPSLNMIIGPNGSGKSTFVCAVCLGLAGKPEYI
                    *:*::*:: :****   *:  .* **::*****:****:*.*:*:**.  *: :

SmcA    162-GRAKDPGEFVKHGCREASIEIELAKGPGL---------RKNPVISRTIKREGNKSSFTINGKQ
Spr18    65-GRAKEAREFIKYGKNTATIEIEMKYRD-----------DETVTITRQISQD-KSSSFSINREA
YOL034w  94-GRSKKVEDFIKNGQDVSKIEITLKNSPNVTDIEYIDARDETIKITRIITRSKRRSDYLINDYQ
            **:*.  :*:* *   :.*** :                :.  *:* *.:. . *.: **

SmcA     216-ASLAQVKKFAQSFAIQIDNLCQFLPQDRVSEFAAL
Spr18    116-CATSSITSLMDTFNVQLNNLCHFLPQDRVAEFAQL
YOL034w  157-VSESVVKTLVAQLNIQLDNLCQFLSQERVEEFARL
              : : :..:   : :*::***:**.*:** *** *
```

SMC_C

```
SmcA     981-KLEELVKSISDAFSDSFARIGCAGQVTLDKAEDEEGPNGEPGGSNFDQWSIQIQVKFRENEN
Spr18    897-KLEENVQCISDRFSKGMSGMGYAGEVRLGKSDD------------YDKWYIDILVQFREEEG
YOL034w  928-KLDDIVSKISARFARLFNNVGSAGAVRLEKPKD------------YAEWKIEIMVKFRDNAP
             **:: *. **  *:  :  :* ** * * *..*            : :* *:* *:**::

SmcA    1043-LSILDSHRQSGGERAVSTIFYLMALQSLSASPFRVVDEINQGMDPRNERMVHGRLVDIA
Spr18    947-LQKLTGQRQSGGERSVSTIMYLLSLQGLAIAPFRIVDEINQGMDPRNERVVHRHIVNSV
YOL034w 978-LKKLDSHTQSGGERAVSTVLYMIALQEFTSAPFRVVDEINQGMDSRNERIVHKAMVENA
             *. * .:******:***::*:::** :: :***:*********.****:**   :*: .

SmcA     1102-CAPARNGGGGQYFLITPKLLSGLVY
Spr18    1006-CDNAVS----QYFLVTPKLLPDLTY
YOL034w  1037-CAENTS----QYFLITPKLLTGLHY
              *    .    ****:*****..* *
```

FIGURE 4.—Domain conservation of the predicted *A. fumigatus* SmcA protein. Alignment of SmcA with *S. pombe* Spr18 (Swissprot: accession no. O13710) and *S. cerevisiae* YOL034w (accession no. NP_014608) is shown for the amino- (SMC_N; 50% identity, 70% similarity over 150 amino acids) and carboxy- (SMC_C; 42% identity, 61% similarity over 232 amino acids) terminal domains. Conserved amino acids between the three homologs are marked by a star while similar amino acids are indicated by dots on the bottom lines. The SGG and DE motifs that are conserved in all SMC proteins are in boldface type.

loidization medium (data not shown). Three transformants (190, 226, and 333) displayed variable rearrangements of ppyrG that prevented analysis of flanking genomic DNA regions. Two others (328 and 340) had similar rearrangements of ppyrG but the length of the nucleotide sequences obtained by semirandom PCR was sufficient to reveal genomic sequences located at the junction with rearranged ppyrG. Comparison of these genomic sequences to the wild-type *A. fumigatus* genome sequence showed that the plasmid integration had led

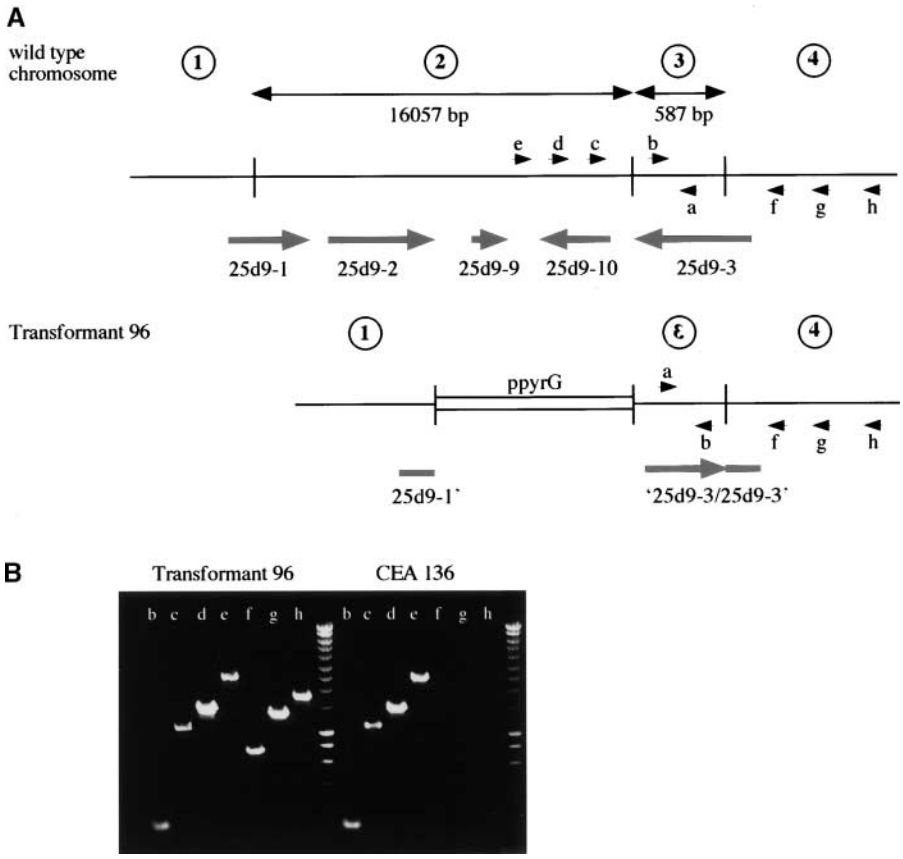

FIGURE 5.—Structure of genomic DNA at the site of integration of ppyrG in *A. fumigatus* transformant 96 of strain CEA136 and at the corresponding locus in a wild-type *A. fumigatus* diploid strain. (A) Map of the proposed organization of genomic DNA at the mutant locus in transformant 96 and at the wild-type locus. Plasmid ppyrG is indicated as an open box. The genomic organization of coding regions is indicated by arrows and numbers. The approximate location of the primers used to analyze the genomic structure in wild-type and mutant strains is indicated by arrowheads. (B) PCR analysis of wild-type diploid strain CEA136 and transformant 96 with primer 96-a in combination with appropriately spaced primers (96-b to 96-h). One wild-type copy of the locus is present in the diploid transformant 96, as shown by the amplification of fragments of identical size to those obtained with CEA136 genomic DNA when primers 96-b, -c, -d, and -e are used. The nature of the inversion is revealed by the occurrence of PCR products with primers 96-f, -g, and -h in transformant 96 only.

to minor genomic DNA deletions in these two transformants (42 and 7 bp, respectively). Finally, analysis of the two remaining transformants (176 and 336) showed that deletions of at least 12.7 and 36.9 kb, respectively, had occurred at the site of integration of ppyrG. We conclude that the frequency of chromosomal or plasmid rearrangements in transformants is independent of their phenotype on selective haploidization media.

DISCUSSION

The parasexual cycle of *A. fumigatus*: The first description of a parasexual cycle in *A. fumigatus* was by Stromnaes and Garber in 1963. Few applications of parasexual genetics have been reported for this organism since (Levadoux *et al.* 1981), although parasexual genetics has proved valuable for genetic mapping and evaluating whether cloned genes are essential in the model species *A. nidulans* (Clutterbuck 1992; Momany and Hamer 1997; Reoyo *et al.* 1998). Here, we describe the establishment of *A. fumigatus* stable diploids that can be used in transformation experiments and for parasexual genetics. Using the parasexual cycle, we showed that the *AfFKS1* gene encoding the catalytic subunit of the β-(1,3)-glucan synthase is essential for the growth of *A. fumigatus*. By examining the formation of cals by heterozygous diploids plated on selective haploidization medium, we screened for mutations lying in genes essential for *A. fumigatus* growth. We analyzed a collection of 544 heterozygous diploids obtained by insertional mutagenesis. Of these mutants, 4.5% produced a cal similar to that observed for *AfFKS1/Affks1* strains and, therefore, presumably harbor a mutation that inactivates an essential gene.

Recent results (Bradshaw *et al.* 2001) using *A. nidulans* show that it is possible to recover poorly growing haploid progenies with a null mutation in cytochrome *c* through cleistothecia originating from a transformed heterokaryon, but not through parasexual genetics. This suggests that parasexual genetics prevents the appearance of haploid strains with a strong decrease in their fitness. Approximately 7% of the *A. fumigatus* mutants in our collection display an ambiguous phenotype (reduced growth, absence of sporulation) upon growth on selective haploidization media (data not shown). Further studies will be needed to understand the requirements to produce haploid mutant spores during the parasexual cycle.

DNA rearrangements following plasmid integration: To generate a collection of insertional mutants of an *A. fumigatus* diploid strain, we used electroporation of a linearized heterologous plasmid. Although our results would suggest that electroporation is the method of choice to generate insertional mutants in *A. fumigatus*, as previously observed in haploid strains (Brown *et al.* 1998), our subsequent characterization of insertional mutants with an apparent single-copy integration showed that integration is associated with significant rearrangements of both plasmid and genomic DNA at the site of insertion. Such rearrangements are not easily detected by hybridization techniques and are a major limitation for the subsequent molecular characterization of tagged mutants.

Natural genomic rearrangements are common in fungi and are the main cause of karyotype variability in populations, playing an important role in adaptation to environment changes (Fierro and Martin 1999). Mutagenesis and DNA-mediated transformation enhance the appearance of genomic rearrangements, as seen in *Neurospora crassa* (Asch *et al.* 1992; Perkins *et al.* 1993), *A. nidulans* (Xuei and Skatrud 1994), *Nectria haematococca* (Kistler and Benny 1992), and *C. albicans* (Barton and Scherer 1994). Nevertheless, insertional mutagenesis with heterologous plasmids has been used extensively in fungi (reviewed in Brown and Holden 1998). To identify *A. fumigatus* virulence factors, random insertional transformants were obtained mainly by electroporation of a haploid strain by Brown *et al.* (2000). The only characterized mutant with severely attenuated virulence has an integration of two copies of the heterologous plasmid in a tail-to-tail configuration without genomic rearrangements (Brown *et al.* 2000). Our characterization of a larger set of transformants indicates that DNA rearrangements following transformation with a heterologous plasmid are common in *A. fumigatus* and were probably underestimated in previous studies. Similar rearrangements also appear associated with the ectopic integration of a homologous plasmid introduced by electroporation (A. Firon and C. d'Enfert, unpublished data).

Analysis of the junctions between ppyrG and *A. fumigatus* genomic DNA suggests common features for illegitimate recombination in *C. glabrata* (Cormack and Falkow 1999) and *A. fumigatus*: absence of microhomologies between plasmid ends and genomic targets, absence of a topoisomerase I consensus next to the genomic target, variable deletions of the plasmid 5′ protruding end, and addition of some bases at the junction that do not originate from plasmid or genomic DNA. This suggests that a nonhomologous end joining process is involved in the integration of heterologous DNA in *A. fumigatus* as proposed in *C. glabrata*. However, illegitimate recombination in *C. glabrata* and in *S. cerevisiae* is not associated with major genomic rearrangements, although genomic duplications and insertions of mitochondrial DNA or small deletions/insertions have been observed at the site of plasmid integration in these species (Schiestl *et al.* 1993; Cormack and Falkow 1999). Further studies on the mechanisms of recombination and DNA repair in *A. fumigatus* and filamentous fungi will be needed to explain the increased frequency and extent of DNA rearrangements that occur in these species.

Identification of essential *A. fumigatus* genes: A collec-

tion of 546 insertional mutants of a diploid *A. fumigatus* strain yielded 25 transformants for which insertion of the transforming DNA seemed to have occurred in a gene essential for *A. fumigatus* growth. As mentioned above, DNA-mediated transformation results in major plasmid and genomic rearrangements. An unambiguous correlation between the observed phenotype and a gene interrupted by the transforming DNA molecule could be obtained for only one transformant. In this transformant, the mutation results in the inactivation of a gene that encodes a member of the SMC protein family that we designate SmcA. Other members of this protein family have been described in lower and higher eukaryotes (Strunnikov and Jessberger 1999). In yeasts, SMC proteins are also essential for cell viability (Lehmann *et al.* 1995; Winzeler *et al.* 1999; Fousteri and Lehmann 2000).

In summary, we have shown that insertional mutagenesis of a diploid *A. fumigatus* strain can be used as a means to identify genes that are required for efficient growth of this fungus. However, electroporation-mediated transformation does not appear to be an appropriate method to generate a collection of *A. fumigatus* insertional mutants that is amenable to rapid molecular characterization. Insertional mutagenesis using *Agrobacterium tumefaciens* T-DNA (de Groot *et al.* 1998) or using heterologous transposons (Daboussi 1996; Kempken and Kuck 1998; Hua-Van *et al.* 2001) may represent suitable alternative strategies to produce *A. fumigatus* insertional mutants. In this regard, we have recently shown that the *Fusarium oxysporum* class II transposable element *impala* (Langin *et al.* 1995; Hua-Van *et al.* 2001) transposes efficiently in *A. fumigatus* without any associated genomic rearrangements and can be used in combination with parasexual genetics to establish a compendium of *A. fumigatus* essential genes (A. Firon and C. d'Enfert, unpublished data).

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