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Identification of Essential Genes in the Human Fungal Pathogen *Aspergillus fumigatus* by Transposon Mutagenesis

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The opportunistic pathogen *Aspergillus fumigatus* is the most frequent cause of deadly airborne fungal infections in developed countries. In order to identify novel antifungal-drug targets, we investigated the genome of *A. fumigatus* for genes that are necessary for efficient fungal growth. An artificial *A. fumigatus* diploid strain with one copy of an engineered *impala160* transposon from *Fusarium oxysporum* integrated into its genome was used to generate a library of diploid strains by random in vivo transposon mutagenesis. Among 2,386 heterozygous diploid strains screened by parasexual genetics, 1.2% had a copy of the transposable element integrated into a locus essential for *A. fumigatus* growth. Comparison of genomic sequences flanking *impala160* in these mutants with that of the genome of *A. fumigatus* allowed the characterization of 20 previously uncharacterized *A. fumigatus* genes. Among these, homologues of genes essential for *Saccharomyces cerevisiae* growth have been identified, as well as genes that do not have homologues in other fungal species. These results confirm that heterologous transposition using the transposable element *impala* is a powerful tool for functional genomics in ascomycota, and they pave the way for defining the complete set of essential genes in *A. fumigatus*, the first step toward target-based development of new antifungal drugs.

Systemic infections by opportunistic pathogenic fungi have become a clinical problem as the number of immunodeficient patients and the development of severely immunosuppressive therapies are increasing (51, 53). The two main causative agents of opportunistic fungal infections are the commensal polymorphic yeast *Candida albicans* and the saprophytic filamentous fungus *Aspergillus fumigatus* (38, 51). Currently available antifungal drugs belong to three main classes: polyenes (e.g., amphotericin B) and azoles (e.g., fluconazole), both of which target fungal membranes (19, 22), and the new echinocandin family (e.g., caspofungin), which targets the enzyme responsible for cell wall β(1,3)-glucan biosynthesis (2, 23). However, systemic fungal infections are still associated with a high mortality, mainly due to the relative toxicity and side effects of antifungal drugs, in addition to often-late diagnosis and the emergence of resistance (15, 19, 49).

A rational approach to increasing our antifungal arsenal relies on the identification of novel targets involved in various aspects of fungal biology (21, 29). Although gene products necessary for virulence are seen as candidate targets (44), no genuine virulence factor in opportunistic fungal pathogens has yet been identified (34, 42). Attractive alternative antifungal targets are to be found among gene products that are essential for fungal growth both in vivo and ex vivo (18, 26). Compendia of essential genes have been obtained for the model eukaryotic microorganism *Saccharomyces cerevisiae* through various approaches (31), including systematic gene inactivation or random insertional mutagenesis in a diploid background followed by analysis of meiotic progenies (20, 47). A set of genes critical for growth of the yeast *C. albicans* has also been defined by using inducible expression of antisense RNA molecules (10). Of the 86 *C. albicans* genes identified, 38% do not have homologues in available databases (10). Differences in essential biological processes between the yeasts *S. cerevisiae* and *C. albicans* highlight the need to study the larger and more complex filamentous fungal genomes in order to reveal species-specific and filamentous fungus-specific targets.

A. fumigatus has become the most prevalent airborne filamentous fungal pathogen (38). Dissemination of *A. fumigatus* occurs by release of asexual spores (conidia) into air (33). They are inhaled daily without major consequences for human health. However, in immunocompromised hosts, *A. fumigatus* can cause a usually fatal infection, termed invasive pulmonary aspergillosis (13, 33, 36). *A. fumigatus* is haploid and devoid of a sexual cycle (33), preventing the application of strategies that use classical genetics to define essential genes. Nevertheless, it has previously been shown that the parasexual cycle, which relies on the chemical haploidization of artificial diploid strains (6, 50), can be used to demonstrate the essential function of *A. fumigatus* genes (17). In this setting, a heterozygous *A. fumigatus* diploid is generated by targeted gene replacement or by random insertional mutagenesis and is subjected to haploidization with or without the selective pressure corresponding to the introduced mutation. The absence of haploid progeny under selective conditions only is indicative of the inactivation of a gene essential for *A. fumigatus* growth (Fig. 1). By using this approach, it was demonstrated that the *A. fumigatus FKS1* gene, encoding the 1,3-β-D-glucan synthase catalytic subunit, and the *smcA* gene, encoding a member of the SMC (structural maintenance of chromosome) protein family, are essential for *A. fumigatus* growth (17). However, it was also reported that the random insertional mutagenesis protocols currently used for *A. fumigatus*, which rely on integration of a heterologous DNA molecule into the fungal genome (4, 12), lead to frequent

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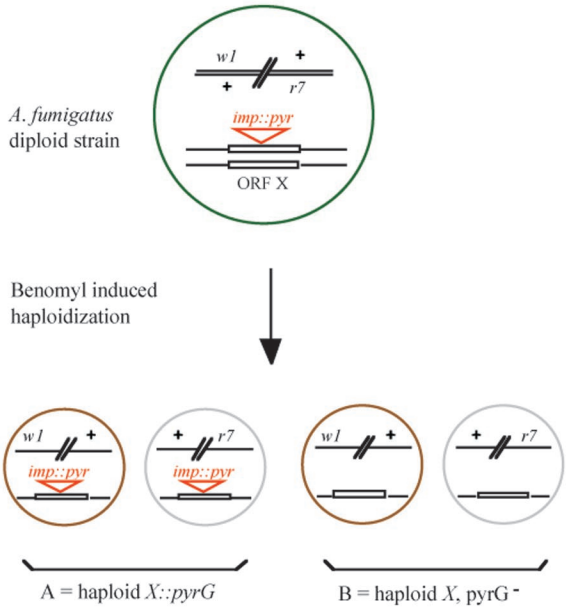

ORF X	Haploid strains	Non-selective haploidization	Selective haploidization
Essential gene	A	-	-
		Growth	No growth
	B	+	-
Non-essential gene	A	+	+
		Growth	Growth
	B	+	-

FIG. 1. Strategy for identification of essential genes in *A. fumigatus*. A stable diploid strain heterozygous for spore color markers (*w1 r7*) is randomly mutagenized with the transposable element *imp160*::*pyrG* (*imp*::*pyr*). During haploidization on a benomyl containing medium, random loss of chromosomes gives rise to two subpopulations of colored haploid conidia (*w1* or *r7*): one bearing the transposon-inactivated allele (population A) and one bearing the wild-type allele (population B). The ability to form haploid progenies on a nonselective haploidization medium and the inability to do so on a selective haploidization medium (without uridine and uracil) leads to identification of mutant strains with an insertion in an essential gene.

genomic rearrangements that hamper a high-throughput analysis (17, 48).

In order to perform genomewide identification of essential genes, we have developed an in vivo transposon mutagenesis system for *A. fumigatus*. Transposons are molecular tools widely used in vitro and/or in vivo for bacteria (30) and yeasts (31, 47), but only very recently have they been applied in the filamentous fungal kingdom (9, 24). In particular, *impala160*, a class II transposable element of the *Tc1-mariner* family (45), has been identified by transposon trapping in the phytopathogenic fungus *Fusarium oxysporum* (32) and has been shown to transpose efficiently in *Fusarium* species (28) as well as in *Aspergillus nidulans* (35) and *Magnaporthe grisea* (52). The results presented here show that *impala160* is also functional in *A. fumigatus* and can be used to generate a collection of random heterozygous diploids. Screening of such a collection by parasexual genetics has resulted in the characterization, without prior sequence information, of *A. fumigatus* genes that are necessary for efficient fungal growth.

MATERIALS AND METHODS

***A. fumigatus* strain construction.** Media and growth conditions were as described previously (17). The *A. fumigatus pyrG niaD* haploid strain CEA113 is a chlorate-resistant derivative of strain CEA17 (11). The *niaD* mutation was confirmed by growth on minimal medium supplemented with different nitrogen sources (0.5 mM sodium glutamate, ammonium tartrate, sodium nitrate, sodium nitrite, or hypoxanthine) as previously described (7). Stable *A. fumigatus* diploids appropriate for transposon mutagenesis were obtained using the following procedure. Insertional mutagenesis of strain CEA17 has led to the isolation of spore color mutants CEA82 and CEA85 (17). The white strain CEA88 and the reddish strain CEA94 are chlorate-resistant derivatives of CEA82 and CEA85 with uncharacterized mutations in a gene involved in the biosynthesis of the molybdene cofactor (*cnx*) and the nitrate reductase gene (*niaD*), respectively. Strains CEA125 (*w1 cnx1 pyrG1*) and CEA129 (*r7 niaD2 pyrG1*) were obtained from strains CEA88 and CEA94 by growth on media containing 5-fluoroorotic acid (1 mg/ml), which selects for *pyrG* mutants. Simultaneous growth of CEA125 and CEA129 on minimal medium with nitrate as the sole nitrogen source yielded heterokaryons that produced grey-green spores similar to those of *A. fumigatus* haploid wild-type strains. This led to the isolation of the stable diploid strain CEA131 (*w1*/+ +/*r7 cnx1*/+ +/*niaD2 pyrG1*/*pyrG1*). A chlorate-resistant derivative of CEA131 that was unable to use nitrate as the sole nitrogen source and was defective at both *niaD* alleles was identified. This strain is referred to as CEA153 (*w1*/+ +/*r7 cnx1*/+ *niaD4*/*niaD2 pyrG1*/*pyrG1*). Spontaneous reversion of strain CEA153 was not observed on minimal medium containing nitrate as the sole nitrogen source.

Transformation and transposition. pNIL160 has been described elsewhere (52). A 2.2-kb *Bam*HI fragment from plasmid ppyrG containing the *A. nidulans pyrG* gene (41) was cloned at the *Nhe*I restriction site in *impala160*, yielding pNIpyr. *Nde*I-digested pNIpyr was introduced into haploid strain CEA113 and into diploid strain CEA153 by electroporation of intact conidia as described previously (4). Briefly, electroporation was carried out with 0.5 μg of linearized plasmid and 5×10^7 conidia in a 0.2-cm electroporation cuvette (Bio-Rad), and cells were subjected to a 1-kV pulse by using a Bio-Rad electroporation device (400 Ω, 25 μF). $pyrG^+$ $niaD^-$ transformants CEA165 (haploid) and CEA225, CEA226, and CEA227 (diploid) were isolated for their high $niaD^+$ reversion frequency and the integration of only one copy of pNIpyr into their genomes. Transposition of *imp160*::*pyrG* occurs after plating of serial dilutions of $pyrG^+$ $niaD^-$ transformant conidia on minimal medium (without uridine and uracil) containing nitrate as the sole nitrogen source and supplemented with 0.02% Triton X-100. Haploid or diploid $pyrG^+$ $niaD^+$ revertants were isolated after 3 days at 37°C. Molecular analysis of *A. fumigatus* strains was performed by Southern blotting techniques after genomic DNA preparation as described previously (17).

Screening for essential genes by haploidization of somatic diploids. Haploidization of *A. fumigatus* heterozygous strains was conducted on selective haploidization medium (rich medium containing 1.2 μg of benomyl [10 mg/ml in dimethyl sulfoxide; Aldrich]/ml) or on nonselective haploidization medium (selective haploidization medium plus uridine and uracil) for 5 days at 37°C (17). Haploid progenies are easily identified by the production of white and reddish sectors after haploidization of grey-green diploid strains.

Sequence determination. Flanking sequence tags (FST) corresponding to genomic sequences bordering the 5′ end of *imp160*::*pyrG* were determined by adaptation of a two-step PCR strategy developed by Chun et al. (5). PCR conditions were as described previously (17), and semirandom primers were used in combination with 5′-end transposon-specific primers (Imp1 [ATGAAGGCG TAAGTTCCTTGC] and Imp2 [GTGTGGAGGAAGAAAGAGC]). Sequencing reactions of gel-purified PCR products were performed by ESGS (Evry, France) using primer Imp2. After removal of transposon sequences, FST were compared by BlastN analysis to the *A. fumigatus* genomic data from The Institute for Genomic Research (TIGR) (www.tigr.org/tdb/e2k1/afu1). The results presented in this study were obtained from the sequence release of 14 November 2001. At this time, the shotgun sequencing of *A. fumigatus* had progressed to 6× sequence coverage (28.7 Mb in 1,578 assemblies of more than 1,000 bp). Analyses of genomic sequences were carried out by Blast searches against public databases (National Center for Biotechnology Information [NCBI] nonredundant protein and expressed genomic tag sequence databases, available at www.ncbi.nlm.nih.gov/BLAST), and the annotation of *S. cerevisiae* genes given in

Results was based on information from Stanford University (*Saccharomyces* Genome Database [SGD], available at http://genome-www.stanford.edu/Saccharomyces) and from the Munich Information Center for Protein Sequences (MIPS) (available at http://mips.gsf.de/proj/yeast/CYGD/db/index.html).

RESULTS

The transposable element *impala* is functional in *A. fumigatus*. A prerequisite for the identification of *A. fumigatus* essential genes through a combination of random insertional mutagenesis and parasexual genetics is that the mutagenic molecules carry a selectable marker (e.g., the *pyrG* gene, encoding orotidine-5′-phosphate decarboxylase and required for uridine-uracil prototrophy), such that diploids can be distinguished for their ability to produce haploid progenies on selective and nonselective haploidization media (e.g., media that differ by the absence or presence of uridine and uracil, respectively) (Fig. 1). Therefore, we constructed plasmid pNIpyr by insertion of the *A. nidulans pyrG* gene between the 3′ end of the transposase-encoding gene and the 3′ inverted terminal repeat of *impala160* in plasmid pNIL160 (52), which contains the *A. nidulans niaD* gene, encoding nitrate reductase, with a copy of *impala160* inserted 10 bp upstream of the translation initiation codon of *niaD* (Fig. 2).

Because of the insertion of *imp160*::*pyrG* into the *niaD* promoter, the *niaD* allele carried by pNIpyr is not functional (Fig. 2). Therefore, introduction of pNIpyr into the *A. fumigatus* haploid *pyrG niaD* strain CEA113 resulted in a haploid $pyrG^+$ $niaD^-$ strain, referred to as CEA165. However, when CEA165 was grown on selective minimal medium with nitrate as the sole nitrogen source, $pyrG^+$ $niaD^+$ revertants were observed at frequencies of 10^{-4} to 10^{-5} (data not shown). Similar results were obtained when plasmid pNIpyr was introduced into *A. fumigatus* strain CEA153, a stable *pyrG niaD* diploid strain that is heterozygous for spore color markers. Three $pyrG^+$ $niaD^-$ diploid transformants (namely, *A. fumigatus* strains CEA225, CEA226, and CEA227) gave $pyrG^+$ $niaD^+$ revertants on selective minimal medium with nitrate as the sole nitrogen source at frequencies of 10^{-5} to 10^{-6} (data not shown).

Southern blot analysis revealed that only one copy of *imp160*::*pyrG* was present in the genome of CEA165, CEA225, CEA226, and CEA227 and that all $pyrG^+$ $niaD^+$ revertants tested ($n = 10$) resulted from the transposition of *imp160*::*pyrG* from the *A. nidulans niaD* promoter to a single, apparently random site located elsewhere in the *A. fumigatus* genome (Fig. 2; also data not shown). Sequence analysis of the *A. nidulans niaD* promoter region in 10 haploid *A. fumigatus* $pyrG^+$ $niaD^+$ revertants revealed that an intact promoter had been restored except for a footprint of 5 bp (CAGTA [$n = 8$], TTGTA [$n = 1$], or CTGTA [$n = 1$]) which results from *impala* excision and does not significantly impair the transcription of the *niaD* gene. Characterization of the genome sequences flanking *imp160*::*pyrG* in $pyrG^+$ $niaD^+$ revertants by sequencing and comparison to public *A. fumigatus* genomic sequences (www.tigr.org/tdb/e2k1/afu1) showed that transposition of *imp160*::*pyrG* occurs at a genomic TA dinucleotide which is duplicated during the integration process, is apparently random without sequence preference (except for the TA), and is not associated with genomic rearrangements (Table 1;also data not shown). These three characteristics, as well as the 5-bp excision footprint, are marks of the transposition of members of the *Tc1-mariner* transposable element family (45) and were previously observed for *impala160* transposition events in *F. oxysporum* (28), *A. nidulans* (35), and *M. grisea* (52).

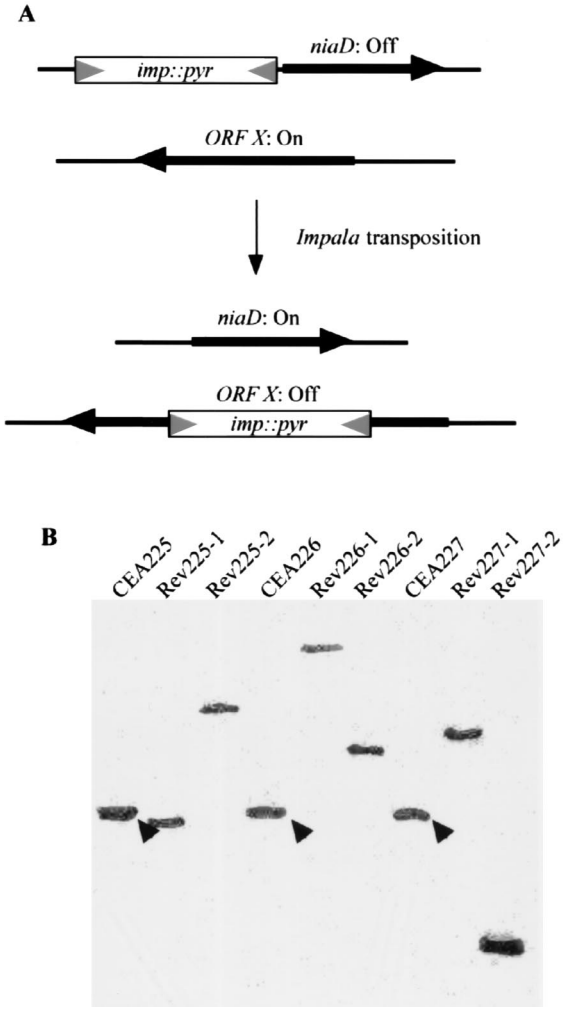

FIG. 2. In vivo random transposon mutagenesis in *A. fumigatus*. (A) Schematic representation of *imp160*::*pyrG* transposition in an *A. fumigatus* strain transformed by pNIpyr. Expression of the nitrate reductase gene (*niaD*) is prevented by the presence of the transposable element *imp160*::*pyG* (*imp*::*pyr*) in the promoter region. Positive selection of transposition events is obtained by selection of nitrate-utilizing revertants, which appear as a result of the excision of *imp*::*pyr* and the restoration of a functional *niaD* promoter. Selection of *imp*::*pyr* reintegration events is ensured by the presence of *pyrG* in the transposable element when transposition events are induced in an *A. fumigatus pyrG* strain and in the absence of uridine and uracil. (B) Southern blot analysis of parental diploid $pyrG^+$ $niaD^-$ transformants (CEA225, CEA226, and CEA227) and diploid $pyrG^+$ $niaD^+$ revertants (Rev225-1 and -2, Rev226-1 and -2, and Rev227-1 and -2). Hybridization with a probe for *imp160*::*pyrG* revealed integration of the transposable element into the promoter of the *niaD* gene in the three parental $pyrG^+$ $niaD^-$ transfomants (arrowheads) and integration at apparently random sites in the genomes of the diploid $pyrG^+$ $niaD^+$ revertants.

Screening for essential genes by haploidization of somatic diploids. The three diploid transformants CEA225, CEA226, and CEA227 were used to generate a collection of 2,386 heterozygous *A. fumigatus* $pyrG^+$ $niaD^+$ revertants by direct se-

TABLE 1. Localization of *imp160*::*pyrG* in *Aspergillus fumigatus* heterozygous diploid strains with a haploid-lethal phenotype

Strain	5′ FST (bp)	Contig[a]		Localization of *imp160*::*pyrG* (bp)
		No.	Size (kb)	
CEA228	97	960	5.1	223
CEA229	435	221	76.1	60969
CEA230	483	1754	6.3	603
CEA231	114	131	27.2	15399
CEA232	92	221	76.1	67069
CEA233	447	164	45.3	40601
CEA234	75	408	71.1	55903
CEA254	199	43	93.1	72592
CEA255	177	408	71.1	53190
CEA256	411	110	207.8	105657
CEA257	201	493	42.2	14886
CEA258	558	190	22.1	7131
CEA259	433	1327	4.7	2928
CEA260	137	493	42.8	36145
CEA261	830	93	53.7	11506
CEA262	281	1366	3.6	1870
CEA263	261	838	9.8	8261
CEA264	119	846	16.7	3278
CEA265	573	573	36.5	13244
CEA266	423	585	39.5	24940
CEA280	900	6	212.7	208370
CEA281	306	792	13.7	1353
CEA282	582	443	89.1	67662
CEA283	339	716	14.3	9480
CEA284	390	652	29.4	18411
CEA285	222	None		

[a] Contigs obtained from http://www.tigr.org, release of 14 November 2001.

lection on minimal medium lacking uridine and uracil. In this setting, the occurrence of revertants should result predominantly from excision and subsequent reintegration of *imp160*::*pyrG*. since transposition events associated with the loss of *imp160*::*pyrG* would result in uridine and uracil auxotrophs which cannot grow on minimal medium lacking uridine and uracil.

Haploidization of the 2,386 heterozygous diploid somatic strains was induced by the destabilizing reagent benomyl (6, 27). Strains CEA225, CEA226, and CEA227 and 97% of 2,386 *pyrG*$^+$ *niaD*$^+$ revertants showed no difference on selective and nonselective haploidization media in two independent tests, indicating that integration of pNIpyr into the genomes of the parental transformants and integration of *imp160*::*pyrG* in the majority of the *pyrG*$^+$ *niaD*$^+$ diploid revertants had not occurred in an essential locus (Fig. 3 and data not shown). In contrast, 73 mutants (3%) did not yield haploid conidia on selective haploidization medium, as indicated by the absence of colored sectors (Fig. 3 and data not shown).

Diploid strains of *A. fumigatus* are hypersensitive to benomyl, and only haploid strains can grow at the benomyl concentration used (1.2 μg/ml) (27). However, transient formation of aneuploids during the haploidization of somatic diploids is often observed. These aneuploids display residual growth on selective haploidization medium and can to some extent overgrow haploid strains with morphological defects (17). In order to identify mutants which could have been retained in our screen because of the slow growth of haploid progenies rather than the lethality of the insertion, ca. 10^6 haploid progenies obtained by growth of the 73 mutants described above on nonselective haploidization medium were tested for the occurrence of *imp160*::*pyrG* by growth on selective medium. For 29 diploid revertants (29/2,386 = 1.2%), neither haploid nor aneuploid *pyrG*$^+$ progeny could be obtained, suggesting that these diploids carry a copy of *imp160*::*pyrG* integrated into a chromosomal locus essential for *A. fumigatus* growth.

Characterization of *A. fumigatus* essential genes. FST corresponding to genomic sequences at the 5′ end of *imp160*::*pyrG* were obtained for 26 of the 29 diploid strains mentioned above. Except for one strain (CEA285), corresponding genomic contigs were identified in the public preliminary sequence data for the *A. fumigatus* genome (Table 1). The genomic sequence was used to design specific primers that were used in standard PCRs to confirm the absence of genomic rearrangements after transposon integration and the occurrence of a wild-type chromosomal locus in each of the diploid revertants tested (data not shown). Similarity searches performed using the BLASTx algorithm identified three main categories of insertional mutants. The first category includes 15 strains with *imp160*::*pyrG* inserted into open reading frames (ORFs) with homologues in other fungal species (Table 2). The second category is composed of three strains with *imp160*::*pyrG* integrated into promoter regions. The third category includes seven strains with *imp160*::*pyrG* integrated into loci without homology to previously identified sequences in public databases.

In 9 of the 15 strains of the first category, *imp160*::*pyrG* is integrated into ORFs with homology to genes previously demonstrated to be essential for *S. cerevisiae* growth (Table 2). These yeast genes encode proteins involved in a broad range of essential biological processes such as protein synthesis (*YGL245W* in CEA257), maturation (*WBP1* in CEA256) and transport (*SRP101* in CEA255), nuclear architecture (*NAR1* in CEA233), RNA processing (*DBP10* in CEA231), nucleotide metabolism (*GUK1* in CEA254), chromatin structure (*RSC9* in CEA259), and cell cycle control (*CDC27* in CEA258). Interestingly, the gene interrupted by *imp160*::*pyrG* in strain CEA258 encodes an 809-amino-acid protein which is not only homologous to the *S. cerevisiae* Cdc27 protein but also shows 72% identity and 81% similarity to the *A. nidulans* BimA protein, a tetratricopeptide repeat motif containing protein which is essential for completion of mitosis, and hence growth, in *A. nidulans* (40, 43).

In six additional haploid-lethal strains, *imp160*::*pyrG* is localized into genes encoding homologues of nonessential *S. cerevisiae* proteins (Table 2). However, two of these insertions interrupt the *A. fumigatus* genes for ribosomal proteins Rpl1 (strain CEA262) and Rpl17 (strain CEA261), respectively, which are duplicated in the yeast genome and are not independently essential, although the double mutation is lethal. Another of these six insertions lies in the *A. fumigatus* homologue of *MSW1* (strain CEA230), encoding the yeast tryptophanyl-tRNA synthetase that is localized to mitochondria. A null mutation in *MSW1* leads to a slow-growth phenotype ("petite" phenotype) in yeast due to defects in mitochondrial protein synthesis and respiration (16, 20). That the *A. fumigatus* homologue of *MSW1* is essential reflects the fact that, like most aspergilli, *A. fumigatus* is a strict aerobe that requires mitochondrial function for growth. In this regard, the search for chemically induced respiration-deficient mutants of *A. nidulans* has resulted only in the identification of conditional mu-

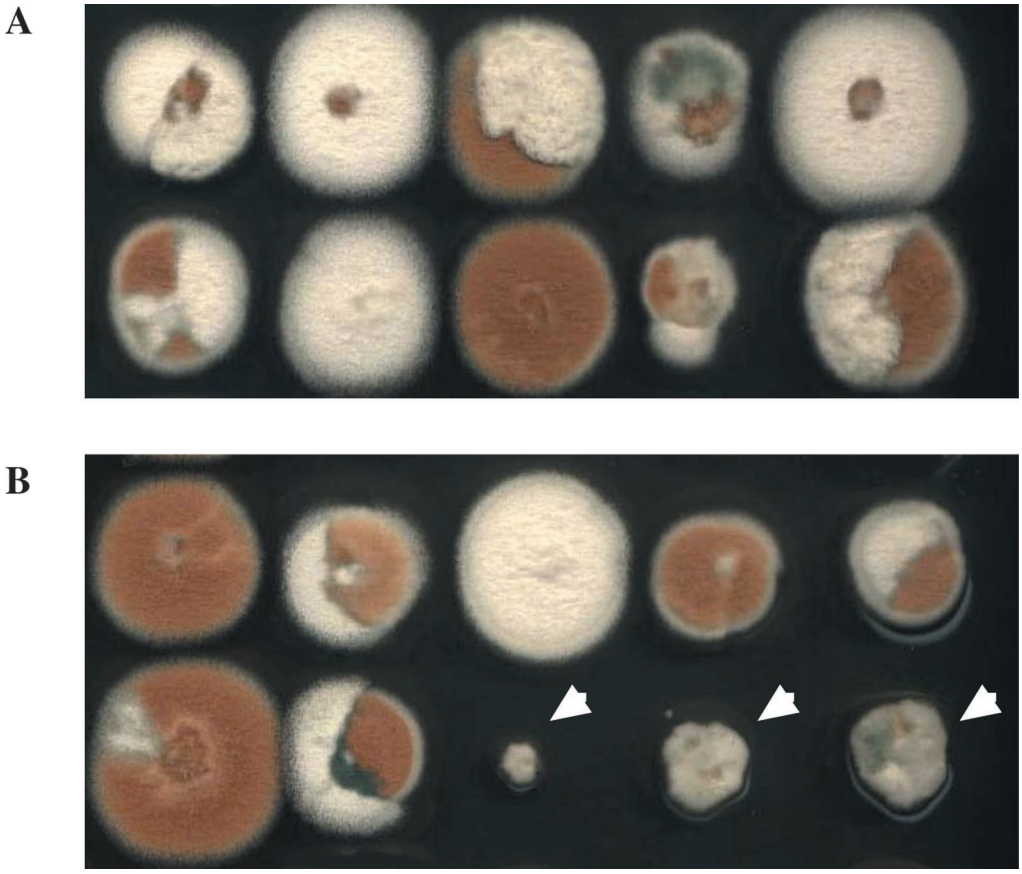

FIG. 3. Parasexual screening. Haploidization of 10 diploid *pyrG*$^+$ *niaD*$^+$ revertants on nonselective (A) and selective (B) media is shown. Random segregation of chromosomes is visualized by the production of differently colored haploid conidia. In the case of plasmid integration in an essential gene, residual growth is observed on selective haploidization medium (arrowheads). For these *pyrG*$^+$ *niaD*$^+$ revertants, haploid spores obtained on nonselective haploidization medium were tested for the absence of the transposable element in order to confirm the essential phenotype.

tants, as would be expected if mitochondrial function is essential for growth in this species also (54). A fourth *imp160*::*pyrG* insertion is located in a homologue of the *S. cerevisiae GOS1* gene (strain CEA263), which is dispensable for vegetative growth but required for ascospore germination at 37°C (39).

Two other genes with significant similarities to dispensable *S. cerevisiae* proteins, namely, Rim11 and Yfl034w, are essential for *A. fumigatus* growth (Table 2, strains CEA228 and CEA264). However, further characterization of the relationships between these *A. fumigatus* and *S. cerevisiae* proteins suggests that they are not orthologues, since the *S. cerevisiae* proteins have closer homologues encoded in the *A. fumigatus* genome than those identified through our insertional mutagenesis screen (Table 2, bidirectional best-hit analysis). Although the homology between these *A. fumigatus* and *S. cerevisiae* proteins is probably indicative of similar biochemical functions, their roles in the cell may differ significantly, as indicated by the difference in their contributions to fungal growth in these two species. In this regard, it should be noted that the *A. fumigatus* homologue of the *S. cerevisiae* protein Rim11 that we have identified is more closely related to the dispensable *Schizosaccharomyces pombe* kinase protein skp1, which might be involved in the control of septation and cytokinesis (46), and that genes involved in septation in *A. nidulans* are known to be essential for sustained growth (25).

The second class of mutants includes three *pyrG*$^+$ *niaD*$^+$ revertants, each with a transposon integration in the vicinity (<200 bp) of the deduced translation initiation codon of a gene which is likely to be essential for *A. fumigatus* growth, based on its homology to an *S. cerevisiae* gene: *RPL14* (strain CEA234), encoding a duplicated ribosomal protein, and *COX10* (strain CEA232) and *HEM15* (strain CEA265), required for heme biosynthesis and respiration (Table 2). In these mutants, it is likely that *imp160*::*pyrG* integration prevents proper expression of these three genes. Yet the possibility that the haploid-lethal defect results from an additional effect on genes divergently transcribed from these intergenic regions cannot be excluded.

Insertions of *imp160*::*pyrG* in the latter seven diploid strains had occurred into an ORF of 2.9 kb (strain CEA229), an ORF of 1.6 kb (strain CEA266), or ORFs shorter than 500 bp without significant homology in public databases (five independent *pyrG*$^+$ *niaD*$^+$ revertants; strains CEA280 to CEA284 in Table 1). This result indicates that approximately 28% (7 of 25) of the essential loci identified through *imp160*::*pyrG* mutagenesis in *A. fumigatus* diploid strains are *A. fumigatus* specific. This is

TABLE 2. *S. cerevisiae* homologues of *A. fumigatus* essential genes

Strain	*S. cerevisiae* closest homologue at the *imp160*::*pyrG* integration locus	Protein length (aa)	Probability (*e* value)	Similarity	BDBH[a]	Essential in *S. cerevisiae*?	Functional category
CEA228	Probable membrane protein (Yfl034wp)	1,073	5*e*-42	55% on 286 aa	No[b]	No	Unknown
CEA230	Mitochondrial tryptophanyl-tRNA synthetase (Msw1p)	379	2*e*-26	54% on 186 aa	Yes	No	Protein synthesis
CEA231	Dead box protein 10 (Dbp10p)	995	*e*-166	55% on 949 aa	Yes	Yes	RNA processing
CEA232[d]	Ferrochelatase (Hem15p)	393	*e*-112	71% on 352 aa	Yes	Yes	Heme biosynthesis
CEA233	Nuclear architecture-related protein (Nar1p)	491	4*e*-59	48% on 465 aa	Yes	Yes	Nuclear architecture
CEA234[d]	Ribosomal protein [Rpl14(a/b)p]	138	1*e*-24	63% on 130 aa	Yes	No	Protein synthesis
CEA254	Guanylate kinase (Guk1p)	187	2*e*-62	81% on 182 aa	Yes	Yes	Nucleotide metabolism
CEA255	Signal recognition particle receptor alpha subunit (Srp101p)	621	*e*-102	62% on 431 aa	Yes	Yes	Protein transport
CEA256	Oligosaccharyl transferase beta subunit (Wbp1p)	430	2*e*-37	46% on 432 aa	Yes	Yes	Protein modification
CEA257	Glutamate-tRNA synthetase (Ygl245wp)	724	0.0	67% on 622 aa	Yes	Yes	Protein synthesis
CEA258	Cell division control protein (Cdc27p)	758	4*e*-71	63% on 304 aa	Yes	Yes	Cell cycle control
CEA259	Remodels the structure of chromatin (Rsc9p)	581	5*e*-29	45% on 414 aa	Yes	Yes	Chromatin structure
CEA260	*S*-Adenosylmethionine decarboxylase (Spe2p)	396	1*e*-54	51% on 462 aa	Yes	Yes	Metabolism
CEA261	Ribosomal protein [Rpl17(a/b)p]	136	5*e*-40	92% on 115 aa	Yes	No	Protein synthesis
CEA262	Ribosomal protein [Rpl1(a/b)p]	255	9*e*-86	86% on 238 aa	Yes	No	Protein synthesis
CEA263	SNARE protein (Gos1p)	223	8*e*-31	60% on 224 aa	Yes	No	Protein transport
CEA264	Serine/threonine protein kinase (Rim11p)	370	*e*-104	77% on 323 aa	Yes/no[c]	No	Cell cycle control
CEA265[d]	Protoheme IX farnesyltransferase (Cox10p)	462	1*e*-45	43% on 341 aa	Yes	No	Heme biosynthesis

[a] BDBH, Bi-Directional Best Hit; *A. fumigatus* closest homologue of the *S. cerevisiae* protein.
[b] *A. fumigatus* orthologue is located on TIGR contig 428.
[c] Several paralogues identified in the *A. fumigatus* genome.
[d] Localization of *imp160*::*pyrG* in the promoter region of the gene (less than 250 bp of the putative translation initiation codon).

in the range of what has been observed in other species in which systematic searches for essential genes have been performed (15 to 20% in *Haemophilus influenzae* [1] and 38% in *C. albicans* [12]). However, while it is likely that the two ORFs inactivated by *imp160*::*pyrG* in strains CEA229 and CEA266 encode functions essential for *A. fumigatus* growth, the basis for the phenotype of the five remaining mutants remains to be investigated.

***impala* transposition characteristics.** The frequency of *imp160*::*pyrG* integrations in loci essential for *A. fumigatus* growth (1.2%) was lower than anticipated. Indeed, nearly 17% of the 6,200 *S. cerevisiae* genes are essential (20). Assuming a similar proportion of essential genes in *S. cerevisiae* and *A. fumigatus* and assuming that the *A. fumigatus* genome (32 Mb) encodes 10,000 genes, it can be estimated that 8 to 10% of the heterozygous diploids should have an integration of *imp160*::*pyrG* resulting in a haploid-lethal phenotype. In an attempt to explain the discrepancy between this estimate and the observed frequency of diploid strains with a haploid-lethal phenotype, 82 insertions of *imp160*::*pyrG* obtained in different backgrounds were investigated (Table 3). As mentioned above, integration of *imp160*::*pyrG* occurred systematically at a TA dinucleotide without any genomic sequence preference, as indicated from the comparison of the 40 nucleotides flanking the TA in these 82 insertions (data not shown). Insertion of the transposable element was also apparently not influenced by the chromosomal location, since the 82 insertions were distributed among 70 contigs ranging from 3.6 to 212.7 kb in size and representing 10% of the genome size (eight contigs were the sites of two integrations, and two contigs were the sites of three integrations) (Table 1 and data not shown).

Although these results were indicative of an absence of sequence preference for *imp160*::*pyrG* integration, the detailed characterization of the loci defined by the 82 insertions suggests that transposition of *imp160*::*pyrG* is not truly random. Indeed, analysis of 28 loci defined by insertions obtained in a diploid or haploid background and having no impact on the morphology or growth of *A. fumigatus* at the haploid stage revealed that a vast majority of these insertions lay in noncoding regions, with 7 located in intergenic regions and 18 located in regions where no ORF of a significant size (>500 bp) could be identified (Table 3). Among these 28 insertions, only 3 were located in ORFs; one of these ORFs encoded a homologue of the *A. nidulans* AmdA protein, which is not essential for *A. nidulans* growth (37), and two encoded proteins without homology in the databases (data not shown). Similarly, insertions that resulted in an altered growth phenotype at the haploid

TABLE 3. Frequency of *imp160*::*pyrG* integration into coding and noncoding regions

Genetic background	Phenotype[a]	No. of revertants analyzed	Localization of *imp160*::*pyrG* (%)		
			Coding region	Promoter region[b]	Other
Diploid	No growth	25	68	12	20
	Altered growth	14	21	43	36
	Normal growth	18	11	33	56
Haploid	Altered growth	15	20	20	60
	Normal growth	10	10	10	80

[a] For diploid revertants, growth after haploidization under selective conditions.
[b] Defined as an insertion of *imp160*::*pyrG* localized less than 500 bp from a putative translation start codon.

stage were mostly located in noncoding regions (20 of 29), although in this case a higher frequency of insertions in ORFs and promoter regions was observed (Table 3). Interestingly, an insertion was found located 237 bp upstream of the deduced initiation codon of a gene that encodes a homologue of an essential *S. cerevisiae* tRNA seryl transferase, suggesting that in this case the altered growth phenotype might result from an effect of the *imp160*::*pyrG* insertion on the transcription of this gene (data not shown). The data obtained from the characterization of *imp160*::*pyrG* insertions that result in a haploid-lethal phenotype (see the preceding section) contrast markedly with these observations, since in that group, a majority of insertions were located in ORFs (17 of 25). Taken together, these data suggest that *imp160*::*pyrG* has a tendency to insert preferentially into noncoding regions, generating phenotypically silent mutations, as already observed for other transposons (8). However, the proportion of integration of *imp160*::*pyrG* into an ORF is directly correlated to the stringency of the screen, as illustrated by our screening for essential genes by parasexual analysis, which enriches for insertions that lie predominantly in ORFs.

DISCUSSION

The goal of this study was to develop an efficient procedure for the identification of the entire set of essential genes in the most prevalent human filamentous fungal pathogen, *A. fumigatus*. Previously, it was demonstrated that the combination of insertional mutagenesis in somatic diploid strains and parasexual genetics can be used to determine whether a particular gene is essential for *A. fumigatus* or not, as illustrated by the essential nature of the *A. fumigatus FKS1* gene, encoding the 1,3-β-D-glucan synthase catalytic subunit (17). However, due to the large number of genes encoded in the *A. fumigatus* genome (estimated at 10,000) and the relative poor efficiency of homologous recombination in this species, a random approach is necessary to define the set of essential genes. A random approach is also advantageous because no prior information on sequences or functions is necessary. Unfortunately, random insertional mutagenesis tools developed in *A. fumigatus* have so far been based on the integration of heterologous plasmid DNA into genomic DNA (3, 4), a process that results in significant genomic rearrangements that hamper a high-throughput analysis (17).

The functionality of the autonomous transposable element *impala160* (32) in different filamentous fungi and its ability to sustain genetically engineered modifications (28, 35, 52) has led us to evaluate this heterologous in vivo transposition system in *A. fumigatus.* Our results demonstrate that an engineered *impala160* element (*imp160*::*pyrG*) is active in both haploid and diploid strains of *A. fumigatus* and that the characteristics of the transposition process are identical to those observed after *impala160* transposition in a *Fusarium* sp. (28), *M. grisea* (52), and *A. nidulans* (35). These results are in agreement with the proposed idea that transposition of members of the *Tc1-mariner* family of transposable element occurs through a conserved mechanism which is independent of host-specific factors (45).

Ideally, a random insertional mutagenesis tool should combine several characteristics: ease in the production of insertional mutants, absence of rearrangements associated with insertions, and random distribution of insertions along the genome. A major advantage of heterologous transposon mutagenesis compared to plasmid-based mutagenesis lies in the absence of DNA rearrangements, allowing rapid characterization of tagged loci. Moreover, the structure of the transposable element *imp160*::*pyrG* allows rapid generation of a large collection of insertional mutants from a single parental strain, since it can be positively selected for both excision (nitrate utilization) and integration (uridine-uracil prototrophy). However, our results show that, although integration of the *imp160*::*pyrG* element seems random at the nucleotide and chromosomal localization levels, this transposable element has a tendency to insert in noncoding regions, and therefore its mutagenesis potential is lower than expected. Li Destri Nicosia et al. (35) have used *impala* in *A. nidulans* to generate more than 10^4 *niaD*$^+$ revertants. Upon visual screening of this collection, only two spore color mutants were identified. The nature of the spore color mutation in one of these mutants has been shown to be the consequence of the integration of the transposable element in the *yB* gene (35). The low frequency of visible mutations after *impala* transposition in *A. nidulans* was proposed to be due to the propensity of *impala* to land outside ORFs (35). We have also observed a low frequency of morphological mutations after transposition of *imp160*::*pyrG* in a haploid *A. fumigatus* strain (15 strains out of 10^6 *pyrG*$^+$ *niaD*$^+$ revertants). The characterization at the sequence level of the loci where *imp160*::*pyrG* is integrated in these strains, as well as in 42 additional *pyrG*$^+$ *niaD*$^+$ revertants which display altered or normal growth in the haploid state, confirms that *imp160*::*pyrG* inserts preferentially into noncoding regions. Despite this integration bias, *impala* was used efficiently in *M. grisea* to identify pathogenicity genes (52). In the sample analyzed, the frequency of nonpathogenic mutants obtained by transposition of *impala160* was similar to that obtained by plasmid-based transformation. Moreover, *impala* integrations were observed in ORFs, and a linkage between the transposon insertion and the loss of pathogenicity was observed in most instances, which is not the case when nonpathogenic mutants of *M. grisea* obtained by DNA-mediated transformation are analyzed (52). Our results obtained using diploid strains of *A. fumigatus* confirm the usefulness of *impala* for generating tagged mutations predominantly in genomic coding sequences after a highly selective screen.

The analysis of 2,386 *A. fumigatus* diploid insertional mutants enabled us to identify 20 previously uncharacterized essential genes, 6 of which could not have been predicted as essential for *A. fumigatus* growth based on studies in other fungal species. The correlation between the growth characteristics of each mutant and the nature of the mutated genes supports the idea that genes genuinely essential for *A. fumigatus* growth can be identified among diploid heterozygous mutants. Furthermore, the identification of essential genes involved in very diverse cellular functions, from protein synthesis to cell cycle control, suggests that the screen that we have performed is not biased for a certain type of cellular function and that scaling it up should result in a wide compendium of *A. fumigatus* essential genes. That some of the homologues of nonessential yeast genes can be essential in *A. fumigatus* might be explained by their implication in additional essential path-

ways and/or by differences in the importance of similar biological pathways to the biology of *A. fumigatus* and other lower eukaryotes. For instance, we have identified several genes encoding components of the mitochondria which are essential in aspergilli, which are strict aerobes, but not in *S. cerevisiae*. The concordance of gene essentiality in different microorganisms is limited, and this fact is illustrated by a recent report by Elitra Pharmaceuticals (San Diego, Calif.), which has used a genome-wide approach to identify essential genes conserved both in *S. cerevisiae* and *C. albicans* (26). Although the detailed results are not available, the investigators report that about half of the *C. albicans* genes that are orthologues of *S. cerevisiae* essential genes are not essential for *C. albicans* growth (26). This limited concordance between microorganisms highlights the need to work directly with relevant pathogenic species and is in favor for the systematic search of essential genes on a random basis. We have now under way a large-scale genomic approach for the identification of essential *A. fumigatus* genes by automation of the strategy using *impala* transposition and parasexual genetics. This represents an important step toward a better understanding of the biology of filamentous fungi and the identification of potential targets for novel antifungal agents. The definition of essential genes will be a functional complement to the *A. fumigatus* genomic sequence (14).

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