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Aspergillus fumigatus C-5 Sterol Desaturases Erg3A and Erg3B: Role in Sterol Biosynthesis and Antifungal Drug Susceptibility

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Two *erg3* genes encoding C-5 sterol desaturase enzymes (Erg3A and Erg3B) in *Aspergillus fumigatus* were characterized with respect to their nucleotide sequences and null mutant phenotypes. Targeted disruption of the *erg3A* and *erg3B* genes and a double gene knockout, *erg3A*$^-$ *erg3B*$^-$, showed that they are not essential for *A. fumigatus* viability. Mutant phenotypes clearly showed that Erg3B is a C-5 sterol desaturase, but no apparent role for Erg3A in *A. fumigatus* ergosterol biosynthesis was found. Susceptibility to amphotericin B, itraconazole, fluconazole, voriconazole, and ketoconazole was not altered in isolates in which *erg3A* and *erg3B* were knocked out alone and in combination.

Aspergillus fumigatus has become the most prevalent airborne fungal pathogen in developed countries, causing allergic diseases, fungal balls, and fatal invasive aspergillosis (27). Furthermore, the incidence of aspergillosis has increased in recent years, due primarily to an increase in immunocompromised patients, particularly those with acute leukemia and bone marrow or solid organ transplants (25).

The majority of *A. fumigatus* isolates are susceptible in vitro to itraconazole, voriconazole, and amphotericin B (AmB) (16, 25); however, isolates with itraconazole MICs of >8 μg/ml have already been well documented and categorized as resistant (4, 9, 12, 25, 33). In addition, those isolates with high MICs have been correlated with poor clinical outcomes in both animal models and patients (10, 11). In contrast, high MICs to AmB are uncommon, although therapeutic failures with this polyene are frequently reported (22).

Azole drugs inhibit the 14-α sterol demethylase (Erg11/Cyp51) in the ergosterol biosynthesis pathway (Fig. 1). In *A. fumigatus*, azole drug resistance has been matched with different types of resistance mechanisms: (i) alteration of the cellular target, such as mutations that decreases the affinity of the enzyme for azole compounds, and (ii) resistance due to altered drug transport (12, 28, 31, 35, 44). In addition, other resistance mechanisms related to modification of other enzymes involved in ergosterol biosynthesis have been described for yeasts. One such enzyme is the C-5 sterol desaturase encoded by *erg3*. Mutations or inactivation of the *erg3* gene in *Saccharomyces cerevisiae* have been associated with azole and polyene drug resistance (3, 20, 44). The sterol composition of *Candida albicans* clinical isolates exhibiting azole and amphotericin B resistance has shown an accumulation of ergosta-7-22-dienol, ergosta-7-enol, and episterol, which are features of the absence of C-5 sterol desaturase activity (23, 24). Furthermore, the loss of function of the *erg3* gene in *Candida dubliniensis* has been described as the primary mechanism of generated itraconazole resistance in vitro (37). Also, a reduction of the expression of the *erg3* gene in *Candida lusitaniae* has been found in two amphotericin B-resistant isolates (50). However, other authors have reported that *C. albicans* isolates with *erg3* deleted and *Candida glabrata erg3* null mutants remain susceptible in vitro to amphotericin B (15, 43). Despite all this previous work on yeasts, no study has been done on *A. fumigatus*. This work describes the identification of three genes in *A. fumigatus (erg3A, erg3B,* and *erg3C*) encoding putative C-5 sterol desaturases and the construction of single (*erg3A* and *erg3B*) and double (*erg3A*$^-$ *erg3B*$^-$) mutant strains lacking functional copies of the genes and their phenotypic characterization.

MATERIALS AND METHODS

Strains. The CM-237 strain of *A. fumigatus* (mold collection of the Spanish National Center for Microbiology) was used throughout this work (30). The fungi were grown at 37°C in GYEP (2% glucose, 0.3% yeast extract, 1% peptone), potato dextrose agar (Oxoid, Madrid, Spain), RPMI (Angus; Oxoid), or minimal medium (6). For propagation of plasmids, *Escherichia coli* strain JM109 was grown in Luria-Bertani (LB) medium (41) supplemented with ampicillin (100 μg/ml).

Cloning and DNA sequencing. The *erg3* fragments were PCR amplified and cloned into the pGEM-T Easy vector system (Promega, Madrid, Spain). DNA inserts of recombinant plasmids were sequenced with the BigDye terminator cycle sequencing ready reaction system (Applied Biosystems, Madrid, Spain) according to the manufacturer's instructions. Sequence analysis was performed on an ABI Prism 377 DNA sequencer (Applied Biosystems), using the sequencing facilities available in the Genomics Department at the Instituto de Salud Carlos III, Majadahonda, Madrid, Spain.

Primer design and PCR conditions. The initial sequence used was a 775-bp sequenced tag (af51C7) from an *A. fumigatus* clone library (ID3355) that was obtained as a gift from J. P. Latge (Pasteur Institute, Paris, France). This sequence showed high homology with different C-5 sterol desaturases encoded by *erg3*.

Primers designed to cover the full genomic sequence from each gene were used to PCR amplify each of the corresponding sequences from the *A. fumigatus* CM-237 genomic DNA (Table 1). All the primers used in this study were synthesized by Sigma Genosys (Madrid, Spain). PCRs were carried out in a 50- to 100-μl volume containing 1× PCR buffer (Applied Biosystems); 2 mM $MgCl_2$ (Applied Biosystems); 250 μM each of dATP, dGTP, dCTP, and dTTP (Applied Biosystems); 1 μM of each primer; 2.5 units of *Taq* DNA polymerase (Applied

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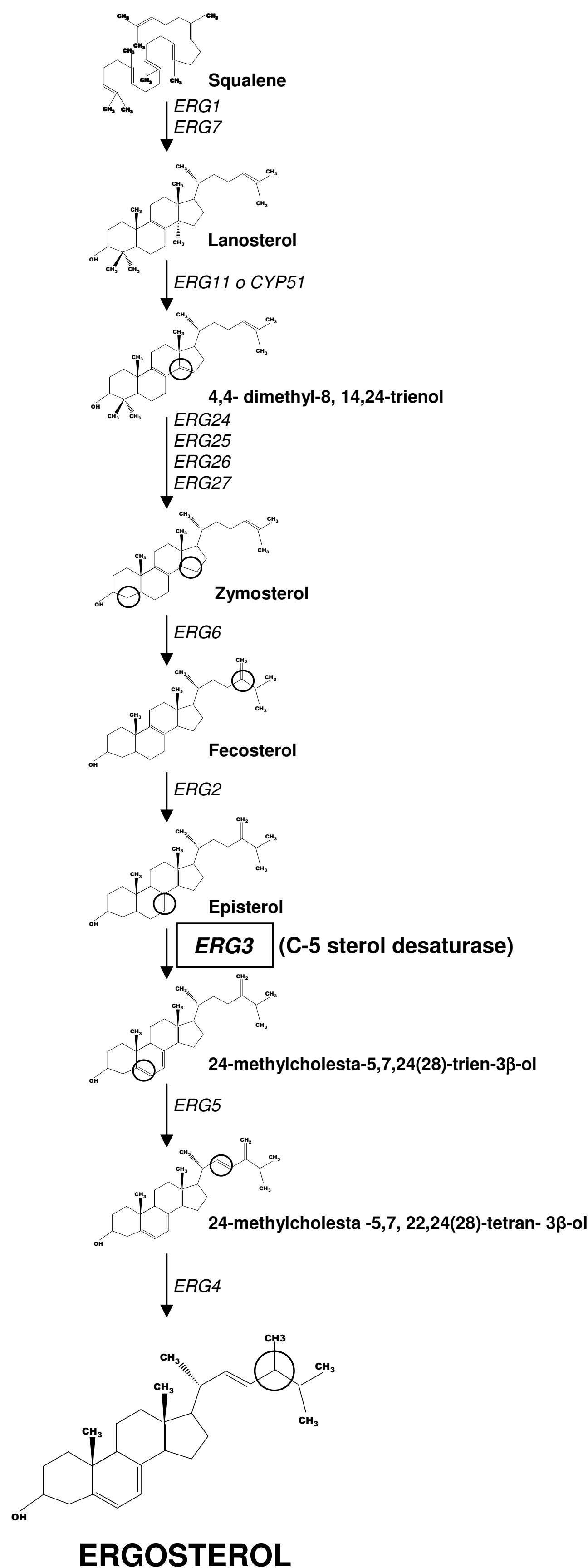

FIG. 1. Ergosterol biosynthesis pathway in *S. cerevisiae* (14, 17).

Biosystems); and 25 to 50 ng of *A. fumigatus* genomic DNA. Amplification was performed in a thermal cycler (GeneAmp PCR system 9700; Applied Biosystems) for one cycle of 5 min at 94°C and then 30 cycles of 30 s at 94°C, 45 s at the necessary melting temperature, and 1 to 2 min at 72°C, followed by one final cycle of 10 min at 72°C. The PCR products were analyzed by electrophoresis on agarose gels.

RNA isolation and RT-PCR. Total RNA was extracted from the *A. fumigatus* CM-237 strain, and reverse transcription (RT) reactions were performed (30). Amplification of cDNAs was carried out using the LightCycler PCR system (Roche Diagnostics, Madrid, Spain). The primers ERG3F and ERG3Z were used to amplify the cDNA from *erg3A*, and primers ERG3B1 and ERG3B2 were used for *erg3B* (Table 1). The RT-PCR products were resolved by electrophoresis on 1.4% agarose gels and were purified for sequencing. Also, the primer set Tub1-Tub2 (Table 1) was used for amplification of the *A. fumigatus* β-tubulin housekeeping gene (*Tub1*) (GenBank accession number AY048754). LightCycler PCRs were set up with Fast-Start DNA Master SYBR Green (Roche Diagnostic). Each assay was repeated on three separate days. PCR efficiencies were calculated from the curve slopes given by the LightCycler software (Roche Diagnostic).

DNA isolation and hybridization. Genomic DNAs from the *A. fumigatus* strains were obtained using a rapid extraction procedure (46) and digested with different restriction enzymes. Southern analysis was performed as previously described (30). Probes for *erg3A* and *erg3B* were obtained by restriction digestion of the appropriate clones. The desired fragments were fractionated in 0.7% low-melting-point agarose gels and excised for labeling with a random-prime DNA labeling system (ECL; Amersham Biosciences) according to the manufacturer's instructions.

Computer analyses. The *A. fumigatus* genome databank at The Institute for Genomic Research (TIGR) was used to search for *A. fumigatus* sequences (http://tigrblastn.tigr.org/ufmg/data/blastn-a_fumigatus). The amino acid sequences of putative C-5 sterol desaturase gene fragments were deduced from nucleotide sequences by using the MapDraw software package and analyzed using the MegAlign software package (DNAstar, Inc., Lasergene, Madison, Wis.). The BLASTp program was used to search for homologous sequences of known proteins in the GenBank database. Multiple amino acid alignments were derived by CLUSTAL analysis, and dendrograms were generated by the unweighted pair-group method using arithmetic averages (19). The final phylogeny was produced by applying the neighbor-joining method (40) to the distance and alignment data.

Molecular cloning and construction of disruption vectors. The full coding sequence of *erg3A* of *A. fumigatus* was PCR amplified as previously described (with primers ERG3D and ERG3Z) and cloned into a pGEM-T vector system (Promega) to obtain plasmid pUM110. The coding sequence of *erg3B*, plus 1 kb on each side, was PCR amplified with primers ERG3B6 and ERG3B7 and cloned into a pGEM-T vector system to obtain plasmid pUM226. A KpnI restriction site was engineered in the middle of *erg3A* and *erg3B* coding sequences by using PCR site-specific mutagenesis (48) with primers described in Table 1 (ERG3Akpn1 and ERG3Akpn2 for *erg3A* and ERG3Bkpn1 and ERG3Bkpn2 for *erg3B*). The hygromycin B (*hph*) resistance cassette (8) was amplified from plasmid pID621 (kindly provided by D. W. Holden) with primers HygKpn1 and HygKpn2 to add flanking KpnI restriction sites for the construction of the disruption vector. The 1.4-kb *hph* cassette was inserted in the KpnI restriction site of pUM206 to create pUM208. The 1.4-kb *hph* cassette was inserted in the KpnI restriction site of pUM226 to create pUM232. The phleomycin (*phle*) resistance cassette was excised from plasmid pID624 (kindly provided by D. W. Holden) by KpnI digestion and inserted in the KpnI restriction site of pUM226 to create pUM235 (Fig. 2).

***Aspergillus fumigatus* transformations.** *A. fumigatus* transformation experiments were done by electroporation, using a protocol previously described (42) with subsequent modifications (12, 49). Hygromycin B (Sigma, Madrid, Spain) (130 μg/ml) was used for single-transformant selection, and phleomycin (Cayla, Madrid, Spain) (150 μg/ml) was used for double-transformant selection. Mutants were named by a letter(s) from the corresponding gene(s) (A, B, or AB) and number. Genomic DNAs from hygromycin- and phleomycin-resistant transformants and the parental strain were all digested with different restriction enzymes (Amersham Biosciences, Madrid, Spain). Southern analysis was performed to confirm gene target events as previously described (12, 30, 41).

Antifungal susceptibility testing. Broth microdilution susceptibility testing was performed as described in the CLSI document M38-A (5) with minor modifications (7, 36, 39). Itraconazole and ketoconazole (both from Janssen Pharmaceutical S.A., Madrid, Spain), voriconazole and fluconazole (both from Pfizer S.A., Madrid, Spain), and amphotericin B (Sigma Aldrich Quimica, S.A., Madrid, Spain) were tested. Susceptibility tests were performed at least three times with each strain on different days.

TABLE 1. Oligonucleotide primers used in this work

Primer	Orientation, 5′→3′	Sequence	Use
ERG3C	Antisense	GCAGGCGTTTGTAGATGG	Specific for *erg3A*
ERG3D	Sense	ATGGACGTTGTGCTTGAC	Specific for *erg3A*
ERG3F	Sense	ATACCTACCTTCAGTACC	Specific for *erg3A*
ERG3Z	Antisense	CTCAGGAAGTTTTCTTGG	Specific for *erg3A*
ERG321	Sense	GCCAAACAATCAAGGCACAG	Specific for *erg3A*
ERG322	Antisense	GATCTATGGTCGGTTGGTTG	Specific for *erg3A*
ERG3B1	Sense	CGGCACCATGGATATTG	Specific for *erg3B*
ERG3B2	Antisense	AGATGTAGGAGAGGGTTG	Specific for *erg3B*
ERG3B3	Sense	ATACCGTTGCCATTCTATTG	Specific for *erg3B*
ERG3B4	Antisense	CGCAACGGAATACGATATTG	Specific for *erg3B*
ERG3B5	Sense	CGAGGAAGTCGATCAAAG	Specific for *erg3B*
ERG3B9	Sense	ATCCCCATGGAGATTGCG	Specific for *erg3B*
ERG3B10	Antisense	GTCGTGAATCATTACCGTC	Specific for *erg3B*
ERG3B6	Sense	GACAGAGTAGGCACGTAG	Specific for *erg3B*
ERG3B7	Antisense	CCCCTTATCTGGCTGACA	Specific for *erg3B*
ERG3C1	Sense	ATCCTCACGCTGCCATGG	Specific for *erg3C*
ERG3C2	Antisense	GTCGCCGTCGTGAATGAG	Specific for *erg3C*
ERG3C3	Sense	ATGGATGTCGCTCTCGAG	Specific for *erg3C*
ERG3C4	Antisense	TTGAAGTCCCTCACTCCC	Specific for *erg3C*
ERG3C5	Sense	AGGATATAAATACCTCCG	Specific for *erg3C*
ERG3C6	Antisense	CTAATCGTCATGCATATG	Specific for *erg3C*
T-7	Universal	TAATACGACTCACTATAGGGCGA	Clone sequencing
Sp-6	Reverse	ATTTAGGTGACACTATAGAATAC	Clone sequencing
ERG3Akpn1	Sense	CCTTCAGGTACCCGCTC	Specific for *erg3A*
ERG3Akpn2	Antisense	GAGCGGGTACCTGAAGG	Specific for *erg3A*
ERG3BKpn1	Sense	CAAGTTGGTACCGACTCC	Specific for *erg3B*
ERG3BKpn2	Antisense	GGAGTCGGTACCAACTTG	Specific for *erg3B*
HygKpn1	Sense	GGTACCTGATATTGAAGG	Specific for *hyg*
HygKpn2	Antisense	GGTACCTTAACTGGTTCC	Specific for *hyg*
Tub1	Sense	AACCAAATTGGTGCCGC	Specific for *tub1*
Tub2	Antisense	CACGGATCTTGGAGATC	Specific for *tub1*

Antifungal susceptibility was also performed by Etest according to the manufacturer's instructions (AB Biodisk, Solna, Sweden).

Sterol analysis. Total ergosterol of strain CM-237 and its mutants was extracted after 18 h of growth in MM liquid medium at 37°C in an orbital sacker incubator (150 rpm), using the protocol described by Arthington-Skaggs et al. (2). Ergosterol content was analyzed by high-pressure liquid chromatography using a μBondapak C_{18} column (Waters LC Module I plus; Waters Corporation, Madrid, Spain). The quantities of sterols were calculated with Millenium[32] and Millenium[32] photodiode array detector software (Waters Corporation). Each experiment was repeated at least three times.

An aliquot of 200 mg of fungal mat was used for sterol extraction to be analyzed by gas chromatography-mass spectrometry (GC-MS). The extraction was performed as described previously (2), but neutral lipids were extracted twice with 1.5 ml hexane. Sterols were converted into their trimethylsilyl ethers by reaction with *N*,*O*-bis-(trimethylsilyl)trifluoroacetamide (85°C, 60 min). Samples were dissolved in toluene and analyzed by GC-MS with a Trace GC gas chromatograph coupled to a quadrupole mass analyzer Trace MS (Thermo, Manchester, United Kingdom). The GC program and MS operation conditions were the same as described previously (29).

Data analysis. The significance of the differences in MICs and ergosterol contents was determined by Student's *t* test (unpaired, unequal variance). A *P* value of <0.01 was considered significant. Statistical analysis was done with the SPSS package (version 13.0; SPSS S.L., Madrid, Spain).

Accession numbers for deduced proteins used in this work. GenBank accession numbers for deduced proteins used in this work are as follows: *Aspergillus nidulans* I, EAA59846; *A. nidulans* II, EAA57846; *Neurospora crassa* I, EAA33687; *N. crassa* II, EAA33687; *Magnaportha grissea*, EAA46489; *Leptosphaeria maculans*, AAN27998; *Ustilago maydis*, EAK84444; *S. cerevisiae*, P32353; *C. glabrata*, AAB02330; *C. albicans*, AAC99343; *C. dubliniensis*, CAD13131; *Schizosaccharomyces pombe* I, BAA21457; *S. pombe* II, CAA22610; and *Homo sapiens*, NP008849.

Nucleotide sequence accession numbers. The full nucleotide sequences of the *erg3A*, *erg3B*, and *erg3C* genes from *A. fumigatus* determined in this work appear in the GenBank nucleotide sequence database under accession numbers AY616449, AY616450, and AY616451, respectively.

RESULTS

Identification of putative *A. fumigatus* C-5 sterol desaturase (*erg3*) sequences. The af51C7 sequence tag from an *A. fumigatus* strain was used to search the *A. fumigatus* genome databank at TIGR. The submitted sequence showed high percentages of nucleotide identity with three different DNA contigs (contig 790, 98%; contig 846, 67%; and contig 363, 59%). The three contigs, including 1 kb of adjacent sequence on each end, were pulled out using the TIGR facilities. Restriction maps and the deduced protein sequence from each of the three genes were obtained. Within the 1,134-bp sequence of *A. fumigatus erg3A*, there was a deduced open reading frame (ORF) of 335 amino acids. The ORF is interrupted twice by introns, based on the presence of matching consensus splice junctions (18). The sequence of *erg3B* (1,132 bp) can be deduced in an ORF encoding 352 amino acids, interrupted by a single intron (18). The 1,014-bp sequence of *A. fumigatus erg3C* is interrupted twice by intron sequences (18) and can be deduced in an ORF encoding 300 amino acids. The deduced amino acid sequences of the *A. fumigatus* gene fragments were used to carry out a BLASTp sequence similarity search in the Swissprot database of GenBank to identify homologous proteins. The results showed that Erg3A and Erg3B have a variable percentage of identity (from 46% to 89%) compared to a variety of filamentous fungi, yeasts, and human C-5 sterol desaturases. The Erg3C showed enough homology to belong to the Erg3 protein family, although percentages were much lower (36 to 61%). We therefore continued with the analysis of Erg3A and Erg3B.

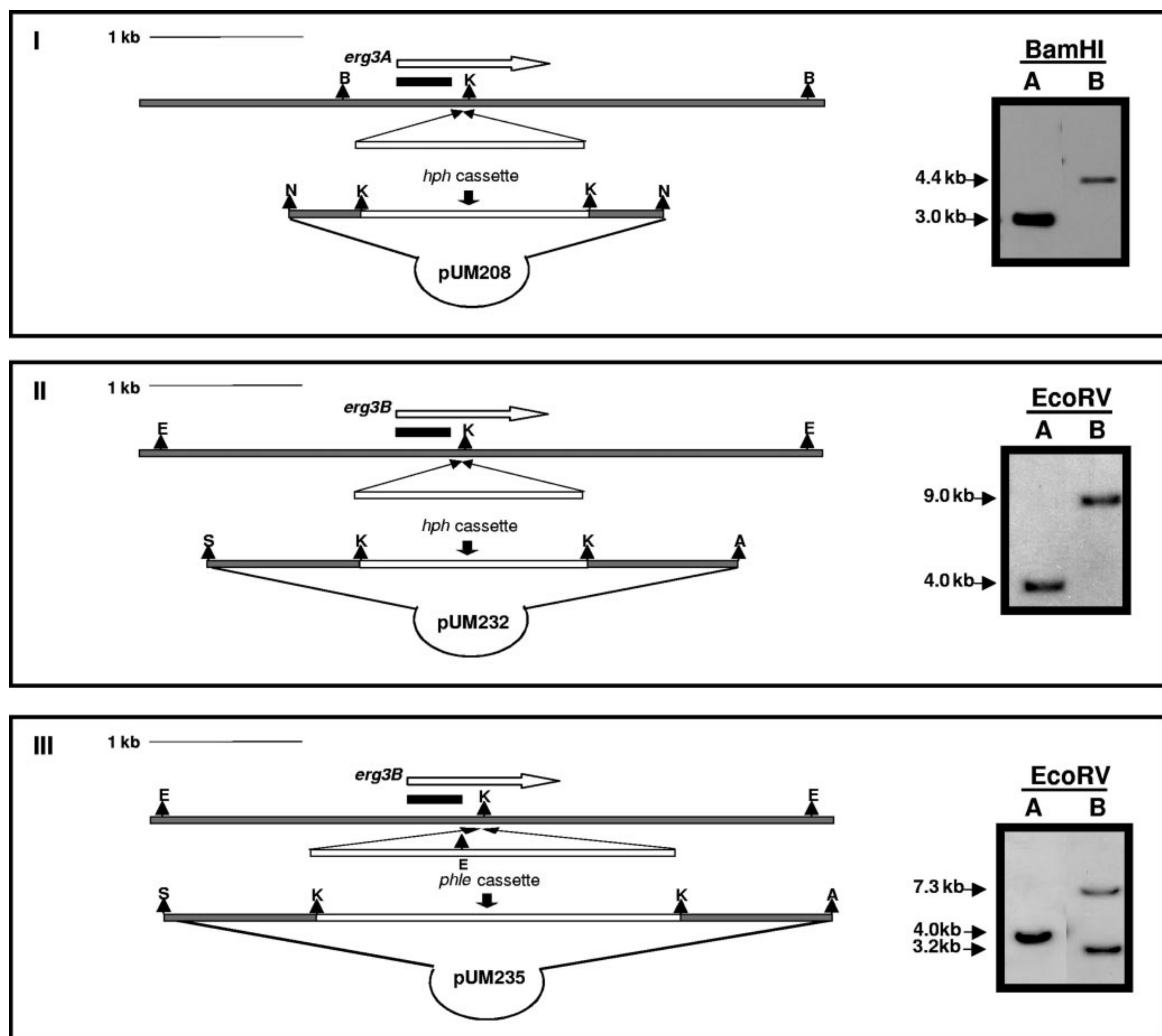

FIG. 2. Diagrams of plasmid constructs used for creating the *A. fumigatus erg3A*-deficient mutant strain (pUM208) (I), the *erg3B*⁻ strain (pUM232) (II), and the double *erg3A*⁻ *erg3B*⁻ mutant (pUM235) (III). Arrows represent the full coding sequences, gray bars represent the genomic sequence, unfilled boxes indicate the hygromycin resistance cassette (*hph*) (I and II) or phleomycin resistance cassette (*phle*) (III), and black bars indicate PCR fragments used as probes. The restriction enzymes used were ApaI (A), BamHI (B), EcoRV (E), KpnI (K), NotI (N), and SacI (S). On the right, Southern hybridization analysis of *erg3A*⁻ single mutant strain CM-A80 (I), *erg3B*⁻ strain CM-B866 (II), and the double *erg3A*⁻ *erg3B*⁻ strain (III) is shown. Genomic DNAs were digested with BamHI and hybridized using the *erg3A* gene probe for CM-A80 (I). CM-B866 (II) and CM-AB118 (III) were detected by digesting the genomic DNAs with EcoRV and hybridizing with the *erg3B* gene probe.

Genomic organization and gene expression. *A. fumigatus* genomic DNAs digested with different restriction enzymes were hybridized with fragments of each gene (*erg3A* and *erg3B*) used as probes. Southern hybridization confirmed that each probe hybridized to a different single band in each case, showing that each gene is present as a single copy in the genome of *A. fumigatus* (data not shown).

RT-PCR showed that both genes were expressed during hyphal growth in submerged culture. RT-dependent products of the expected sizes were amplified for each of the *erg3* genes. Sequencing of each amplified band confirmed the presence of two introns in *erg3A* and one in *erg3B*.

***A. fumigatus erg3A* and *erg3B* full gene sequences and protein analysis.** Specific primers were used to amplify the full genomic sequence for each gene (*erg3A* and *erg3B*) from the *A. fumigatus* CM-237 strain (Table 1). *erg3A* was amplified using primers ERG321 and ERG322, and this product was sequenced with primers ERG3C, ERG3D, ERG3F, and ERG3Z in order to obtain the full sequence on both strands. To amplify *erg3B*, primers ERG3B3 and ERG3B4 were used and the

TABLE 2. Susceptibility testing of *A. fumigatus* wild type and mutants

Strain	Genotype	MIC (μg/ml)[a]				
		AmB	Itraconazole	Voriconazole	Fluconazole	Ketoconazole
CM-237	Wild type	0.25–0.5	0.25–0.5	0.5	>640	4–8
CM-A80	*erg3A* knockout	0.25–0.5	0.12–0.5	0.5	>640	4–8
CM-B866	*erg3B* knockout	0.25–0.5	0.12	0.12–0.5	>640	4–8
CM-AB118	*erg3A erg3B* knockout	0.25–0.5	0.12–0.25	0.25	>640	4–8

[a] Testing was performed three times on different days.

product was fully sequenced with primers ERG3B1, ERG3B5, ERG3B9, and ERG3B10. The inferred 335-amino-acid protein Erg3A of *A. fumigatus* was compared to the other known complete Erg3 amino acid sequences obtained from GenBank. Strong homology was found with other Erg3 proteins from filamentous fungi: *A. nidulans* (II) Erg3 (60%); *N. crassa* (I) Erg3 (57%), and *A. fumigatus* deduced protein Erg3B (56%). Percentages of identity with the other yeast proteins were also very high (*C. albicans*, 46%; *C. dubliniensis*, 47%; *S. cerevisiae*, 46%; and *C. glabrata*, 47%). The inferred 352-amino-acid protein of *erg3B* was also compared to the same Erg3 sequences. A high degree of homology was shown with *A. nidulans* (II) Erg3 (89%), *N. crassa* (I) Erg3 (62%), and *M. grissea* Erg3 (60%). The homologies with yeast proteins were as follows: *C. albicans*, 40%; *C. dubliniensis*, 46%; *C. glabrata*, 47%; and *S. cerevisiae*, 47%.

Generation of *erg3A⁻* and *erg3B⁻* single mutant and *erg3A⁻ erg3B⁻* double mutant strains. We analyzed the functions of *erg3A* and *erg3B* by replacing the chromosomal copies of the genes with mutant versions in which the coding regions had been interrupted by the insertion of drug resistance markers. A hygromycin resistance marker was used for selection of single mutants (*erg3A*$^-$ and *erg3B*$^-$), and a phleomycin resistance marker was used to allow for selection of double mutants (*erg3A*$^-$ *erg3B*$^-$). To generate *erg3A*$^-$ mutants, spores of *A. fumigatus* CM-237 were electroporated using a 3.0-kb linear fragment released from pUM208 by NotI digestion (Fig. 2I). One hundred eighteen hygromycin-resistant transformants were screened by restriction enzyme digestion of genomic DNA and Southern hybridization. One of the transformants tested (CM-A80) appeared to have undergone gene replacement as shown by the shift of a hybridizing DNA fragment in strain CM-237 from 3.0 kb to 4.4 kb (Fig. 2I). Two more enzymes were used for gene replacement confirmation (data not shown).

To produce *erg3B*$^-$ mutants, spores of *A. fumigatus* CM-237 were electroporated using a 3.5-kb linear fragment released from pUM232 by ApaI/SacI double digestion (Fig. 2II). Three hundred sixty-six hygromycin-resistant transformants were screened. Any gene replacement event would be seen by a shift of a hybridizing DNA fragment in strain CM-237 from 4.0 kb to 5.4 kb. One transformant showed a band of 9.0 kb (with disappearance of the 4.0-kb wild-type band), suggesting a disruption of the gene with a tandem integration (Fig. 2II). Two more enzymes were used for confirmation of that specific integration (data not shown), and this was also verified by PCR using primers outside the replaced sequences.

For the construction of the *erg3A*$^-$ *erg3B*$^-$ double knockout mutant, spores of the *A. fumigatus erg3A*$^-$ mutant strain CM-A80 were electroporated using a 4.4-kb linear fragment released from pUM235 (containing the *erg3B* sequence interrupted by the phleomycin resistance marker) by ApaI/SacI double digestion (Fig. 2III). A total of 125 phleomycin-resistant transformants were analyzed by restriction enzyme digestion of genomic DNA and Southern hybridization. One transformant (CM-AB118) appeared to have undergone gene replacement of the interrupted *erg3B* gene, as shown by a shift of a hybridizing DNA fragment in strain CM-A80 from 4.0 kb to two different bands of 7.3 kb and 3.2 kb. As had occurred before, integration of the disruption vector also took place in tandem, including the insertion of one copy of the plasmid (Fig. 2III). Further analysis confirmed that the mutant was hygromycin resistant, and PCR amplification showed that CM-AB118 had retained the genotype of *erg3A*$^-$ disruption.

Morphology of mutant strains. Spores (10^3) of wild-type strain CM-237 and mutant strains were plated in different media to study colony morphology and rate of growth for 72 h (38). No differences were found between the CM-A80, CM-B866, and CM-AB118 mutant strains and strain CM-237. Microscopically, no differences were observed in terms of hyphal or conidial morphology in any of the strains.

Antifungal susceptibility testing. The results of antifungal susceptibility testing are shown in Table 2. The antifungal drug susceptibility profile of the mutants compared with the parental strain (CM-237) showed that there were no differences in susceptibility for any of the antifungal drugs tested. Results obtained by Etest also did not show any significant differences (data not shown).

Sterol composition. The total ergosterol content was analyzed by high-pressure liquid chromatography, showing that this was similar for the wild-type strain CM-237 and the *erg3A*$^-$ mutant (5.99 $\pm$ 0.86 μg/mg and 6.58 $\pm$ 0.29 μg/mg, respectively). However, significant differences in total ergosterol were found for strains CM-B866 (2.02 $\pm$ 0.74 μg/mg) and CM-AB118 (1.75 $\pm$ 0.75 μg/mg). Moreover, the ergosterol UV spectrophotometric profiles for CM-B866 and CM-AB118 did not match with that of the commercial ergosterol, suggesting that other sterols could have accumulated as a result of *erg3B* deletion (data not shown). The wild type and mutants obtained during this study were consequently subjected to GC-MS analysis for the identification of sterol derivatives. The sterols were identified by comparison with known sterol spectra, and the results were expressed as percent area. As summarized in Table 3, ergosterol was the major sterol in the wild-type (CM-237) (73%) and CM-A80 (63%) strains. However, strains CM-866 and CM-AB118 showed a remarkable increase in three sterol fractions: 24-methylcholesta-7,22-dien-3β-ol (39% and 35%, respectively), 24-methylcholesta-7,22,24(28)-trien-3β-ol

TABLE 3. Relative compositions of sterols identified in *A. fumigatus* control strain CM-237 and in the CM-A80, CM-B866, and CM-AB118 mutant strains

Sterol	% in strain:			
	CM-237	CM-A80	CM-B866	CM-AB118
24-Methylcholesta-5,7,9(11),22-tetraen-3β-ol	1.07	3.27	1.20	0.84
24-Methylcholesta-5,8,22-trien-3β-ol (lichesterol)	0.89	0.84	0.06	0.10
24-Methylcholesta-5,7,9,22-tetraen-3β-ol	2.13	5.39	4.67	0.43
24-Methylcholesta-5,7,22-trien-3β-ol (ergosterol)	**73.50**	**63.30**	**11.85**	**17.76**
24-Methylcholesta-7,22-dien-3β-ol	2.12	1.08	**39.14**	**35.73**
24-Methylcholesta-5,7,22,24(28)-tetraen-3β-ol	0.44	0.48	ND	ND
24-Methylcholesta-7,22,24(28)-trien-3β-ol	0.40	0.88	**19.76**	**19.95**
24-Methylcholesta-5,7,24(28)-trien-3β-ol	1.49	1.14	ND	ND
24-Methylcholesta-7,24(28)-dien-3β-ol (episterol)	2.22	3.07	**11.66**	**12.52**
24-Ethylcholest-5,7,22-trien-3β-ol	4.65	4.93	1.48	0.79
4,4,14-Trimethylcholesta-8,24-dien-3β-ol (lanosterol)	2.19	2.43	2.06	1.79
4α,24-Dimethylcholesta-8,24(28)-dien-3β-ol	1.57	2.57	1.59	1.89
4,4,14,24-Tetramethylcholesta-8,24(28)-dien-3β-ol (eburicol)	2.72	3.69	2.32	2.91
4,4,24-Trimethylcholesta-8,24(28)-dien-3b-ol	4.60	6.90	4.20	5.29

[a] Boldface indicates major differences in percentages.
[b] ND, not detected.

(19%), and 24-methylcholesta-7,24(28)-dien-3β-ol (11% and 12%, respectively). The accumulation of non-C-5 desaturated sterols in CM-B866 and CM-AB118 strain was coupled with a decrease of total ergosterol (11% and 17%, respectively).

DISCUSSION

Ergosterol is the major sterol component in fungal membranes and contributes to a variety of cellular functions, including fluidity and integrity of the membranes and the proper function of membrane-bound enzymes (17). This fact makes enzymes involved in the ergosterol pathway attractive targets for antifungal agents. The C-5 sterol desaturase (Erg3) acts towards the end of the ergosterol biosynthetic pathway. It is located in the endoplasmic reticulum, where it catalyzes the introduction of a double bond between C-5 and C-6 in the B ring of the developing sterol molecule, converting episterol to 24-methylcholesta-5,7,24(28)-trien-3β-ol (45, 47) (Fig. 1).

erg3 genes have been isolated from many different yeasts and some filamentous fungi, due to their possible involvement in the study of resistance mechanisms of fungi against azole drugs and polyene compounds (1, 15, 26). The predicted amino acid sequences of *A. fumigatus* Erg3A and Erg3B revealed that they are different proteins and have a high degree of preservation among other C-5 sterol desaturase proteins. Both proteins have identity percentages that are sufficiently high for them to be considered members of the C-5 sterol desaturases family (34). Alignment between both *A. fumigatus* C-5 sterol desaturases and their orthologs in yeasts, filamentous fungi, plants, and humans showed that all have three conserved histidine domains. These domains are present in all known sequences of C-5 sterol desaturases and in a significant class of integral membrane enzymes that includes desaturases, hydroxylases, epoxidases, and acetylases (47). The preserved histidine-rich motifs are thought to contain the active site of the enzyme, being putative iron binding domains in *C. albicans* (32). Furthermore, all of them have the consensus motif for retention of transmembrane protein in the endoplasmic reticulum (21). The enzymatic conversion of lanosterol to ergosterol carries out enzymatic transformations that can occur in a different order in yeasts (14). A comparative study of the genes involved in *Aspergillus* ergosterol biosynthesis (*erg*) has shown the existence of several duplicated genes, including *erg3* (13). The construction of strains with null mutations of genes involved in the biosynthesis of ergosterol and analysis of their sterol profiles could provide valuable information regarding the biosynthesis of ergosterol in *A. fumigatus*. We have identified different *erg3* sequences that could encode C-5 sterol desaturases proteins in *A. fumigatus*. Despite the fact that three putative *erg3* sequences had been identified, we started functional analysis with the Erg3 proteins that showed higher C-5 sterol desaturase homologies. As a result, *erg3A*, *erg3B*, and the combination of both were inactivated in order to study their roles in the *A. fumigatus* ergosterol biosynthesis pathway and their implication in antifungal drug susceptibility.

Functional analysis of Erg3A and Erg3B showed that neither is essential for cell viability and that the two genes are functionally quite different, despite their amino acid homology. Inactivation of the *erg3B* caused the accumulation of non-C-5 desaturated sterols: 24-methylcholesta-7,22-dien-3β-ol, 24-methylcholesta-7,22,24(28)-trien-3β-ol, and 24-methylcholesta-7,24(28)-dien-3β-ol. The lack of any obvious sterol composition alteration in the *erg3A*$^-$ strain implies that Erg3A might not be a C-5 sterol desaturase, although functional compensation by others enzymes (Erg3B and/or Erg3C) could not be ruled out. Alternatively, Erg3A could act in a different phase of the *A. fumigatus* cell cycle or under other growth conditions. To rule out the possibility that Erg3B could be compensating for the lack of Erg3A, we constructed a double mutant strain carrying disruption in genes encoding both proteins (Erg3A and Erg3B). The double mutant (*erg3A*$^-$ *erg3B*$^-$) was indistinguishable from the *erg3B*$^-$ single mutant, including in their sterols profiles.

Despite the differences found in sterol composition between mutants and wild-type strains, no differences in AmB or azole drug susceptibility were detected. These results would dismiss any possibility of Erg3A and Erg3B being involved in the resistance mechanisms against antifungal drugs, at least until *A. fumigatus erg3C*$^-$ and *erg3B*$^-$ *erg3C*$^-$ mutants are analyzed.

In yeast, targeted deletion of *erg3* is always coupled with ergosterol depletion, although discrepancies with respect to AmB/azole susceptibility are seen between different species (20, 37, 43, 50). Since ergosterol is the target for AmB, high MICs to this antifungal would be expected in strains with decreased amounts of this sterol. The results obtained imply that AmB could be targeting other sterol derivatives in the cell, or, as other researchers have reported, that AmB standard antifungal susceptibility testing in *A. fumigatus* does not readily identify AmB resistance (22, 5). Moreover, in *A. fumigatus* deletion of the *erg3* genes does not cause total ergosterol synthesis arrest, and this fact is consistent with the existence of more than one C-5 sterol desaturase enzyme that could compensate for the lack of one or even both Erg3 enzymes. Therefore, the involvement of Erg3C in the biosynthesis of the ergosterol pathway requires research. These experiments are under way in our laboratory.

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