ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, July 2006, p. 2533–2536 Vol. 50, No. 7
0066-4804/06/$08.00+0 doi:10.1128/AAC.00187-06

A Phe389Leu Substitution in ErgA Confers Terbinafine Resistance in *Aspergillus fumigatus*

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Received 10 February 2006/Returned for modification 24 March 2006/Accepted 25 April 2006

Replacement of phenylalanine with leucine at position 391 in squalene epoxidase was identified as being responsible for terbinafine resistance in mutants of *Aspergillus nidulans*. The equivalent mutation was engineered into the *ergA* gene of *Aspergillus fumigatus*, resulting in an F389L substitution that also conferred resistance to this pathogenic mold.

Terbinafine (TRB) is a member of the allylamine group of antimycotics interfering with early-stage ergosterol biosynthesis by inhibiting the enzyme squalene epoxidase (SE) (4, 6, 17). TRB is used routinely to treat superficial mycoses, but it has also been used in the successful treatment of chromoblastomycosis, *Fusarium oxysporum* infection, pulmonary aspergillosis, sporotrichosis, and leishmaniasis (7, 14, 16, 18). Thus, the therapeutic potential of TRB extends well beyond its current use against superficial mycoses, since it has potential clinical efficacy in the treatment of subcutaneous and certain systemic infections. In this study, we have explored a mechanism for TRB resistance in *Aspergillus* by first identifying a resistance-associated mutation within the SE of *Aspergillus nidulans*. The homologous mutation was then introduced into *Aspergillus fumigatus*, generating a similar resistant phenotype.

TRB mutants Terb7 and Terb10 (Table 1) were generated from TRB-sensitive *A. nidulans* strain *pabaA1* by UV mutagenesis (15). These mutants exhibited a high level of resistance to TRB (MIC, ≥8.0 μg/ml) in comparison to TRB-sensitive strain *pabaA1* (15). Genetic analysis indicated that a single gene (designated *tebA*), located on chromosome IV, was responsible for resistance to TRB (15). The position of *tebA* was estimated to be within contig 1.132 because of its proximity to the *pyroA* marker. Analysis of the sequence adjacent to the *pyroA* marker identified a putative open reading frame at positions 42692 to 44221 within the contig designated *A. nidulans* hypothetical protein AN7751.2 (GenBank accession no. EAA61539)

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference(s)
Strains		
pabaA1	TRB-sensitive *A. nidulans* strain	15
TerbA7	TRB-resistant mutant of *pabaA1* isolated after UV mutagenesis	15
TerbA10	TRB-resistant mutant of *pabaA1* isolated after UV mutagenesis	15
ATCC 13073	TRB-sensitive *A. fumigatus* strain	ATCC (Manassas, VA)
TRBR-Ec-F389L	TRB-resistant mutant of ATCC 13073 transformed with pRG47	This study
TRBS	TRB-sensitive strain ATCC 13073 transformed with empty plasmid pRG3-AMA1-HYG	This study
TRBR-F389L	TRB-resistant mutant of ATCC 13073 transformed with pRG48	This study
TRBS	Strain derived from TRBR-F389L after plasmid eviction	This study
TRBS	TRB-sensitive strain ATCC 13073 transformed with pRG49	This study
Plasmids		
pRG3-AMA1	Contains *A. nidulans AMA1* gene	1, 2
pRG3-AMA1-HYG	pRG3-AMA1 containing 4-kb KpnI fragment of *E. coli* HYG resistance gene	This study
pRG46	2.5-kb fragment of *ergA* in pCR-TOPO-TA	This study
pRG47	pRG46 containing point mutation F389L in *ergA*	This study
pRG48	pRG3-AMA1-HYG containing 2.5-kb SmaI fragment of *ergA* from pRG47	This study
pRG49	pRG3-AMA1-HYG containing 2.5-kb SmaI fragment of *ergA* from pRG46	This study

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                                                L251F
S.cerevisiae   228 GSSFVGMSLFNAKNPAPMHGHVILGSDHMPILVYQISPEETRILCAYNSPKVP-----AD
C.albicans     219 GSYFIGLYLKNAELPAKGKGHVLLG-GHAPALIYSVSPTETRVLCVYVSSKPPSAA-NDA
A.fumigatus    208 SSRFWGLELIDTKLPMPYYGHVLLSDN-APILLYQIGTHETRILVDIPENLPSASVKNGG
A.nidulans     232 RSKFWGLELIDAELPQPHYGHVLLEPERPPILIYQIGTHETRILCDIPENLPSASVKNGG
T.rubrum       216 KSKFWGLELIDAKLPIPGHGHVVLGDF-PPILIYQIGEHETRILIDIPDNLPSASVANGG

                          (F389L)
                          (F391L)
                           F402L                F420L    P430S
S.cerevisiae   396 VALYSLFAADSDNLKALQKGCFKYFQRGGDCVNKPVEFLSGVLPKPLQLTRVFFAVAFYT
C.albicans     396 ISLYSLFAADKKPLRILRNGCFKYFQRGGECVNGPIGLLSGMLPFPMLLFNHFFSVAFYS
A.fumigatus    383 QALYSLFAADSAFHLLLAPSSLH-----------------------------------
A.nidulans     385 QALYSLFAADDDNLKALQRGCFQYFSMG--MYETPVSLLGGMLKKPEVLFAHFFTVAFLS
T.rubrum       391 QALYSIFAAGDPNLKVLQRGCFRYFQLG--LIDGPIGLLSGIIRSPLVLLRHFYSVAFLA
                   L398F                              F433S
                  (L393F)
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FIG. 1. Multiple-sequence alignment of fungal ErgA domains with amino acid substitutions that confer resistance to TRB. Amino acid substitutions within regions of the Erg1 (ErgA) protein which confer resistance to TRB in *S. cerevisiae* are shown above the sequences (9, 10). The F389L mutation in the ErgA protein that corresponds to the F391L and F402L substitutions observed in the TRB-resistant isolates of *A. nidulans* and *S. cerevisiae* (respectively) is indicated. The L393F mutation observed in the TRB-resistant *Trichophyton rubrum* clinical isolate is also indicated (13).

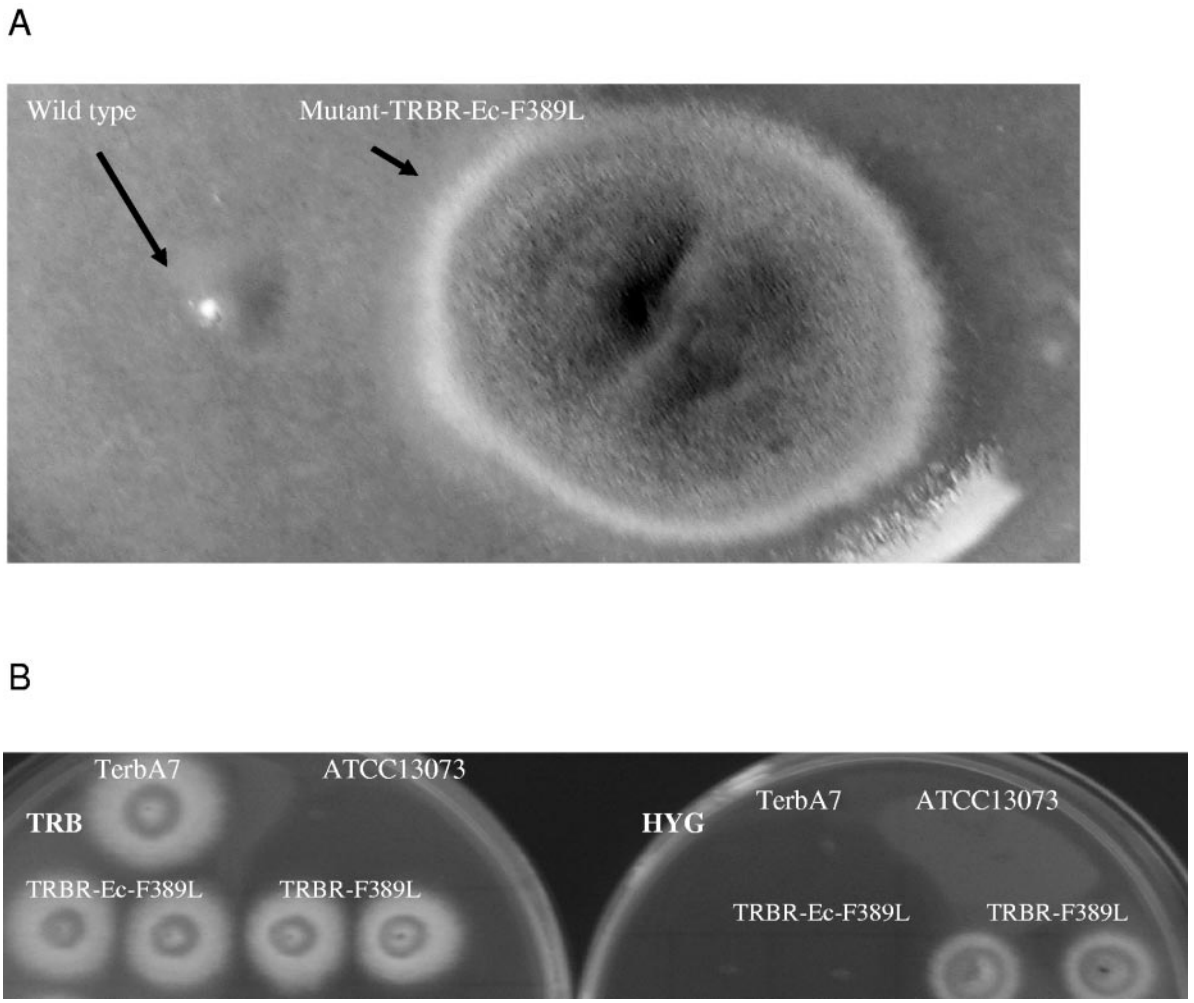

FIG. 2. TRB-sensitive and -resistant isolates of *A. fumigatus*. (A). Growth of strains ATCC 13073 (wild type) and TRBR-Ec-F389L (mutant) upon AM3 with 1.0 μg/ml TRB. (B) Growth of *Aspergillus* upon AM3 with 1.0 μg/ml TRB (left) and upon MM with 200 μg/ml HYG (right). Top, left to right: control strains TerbA7 (TRB-resistant *A. nidulans*) and ATCC 13073 (TRB-sensitive *A. fumigatus*). Bottom, left to right: strains TRBR-Ec-F389L and TRBR-F389L. Strains were grown for 72 h at 37°C.

(http://www.broad.mit.edu/annotation/fungi/aspergillus). The *A. nidulans tebA* sequence displayed 40% sequence identity to the Erg1 proteins from *Saccharomyces cerevisiae* and *Candida albicans*, respectively (GenBank accession no. CAA97201.1 and BAA13565); thus, the *tebA* gene was subsequently referred to as the *ergA* gene (GenBank accession no. DQ391275). Chromosomal DNAs of the Terb7 and Terb10 TRB mutants, which contain the mutations *tebA7* and *tebA10* (15), were extracted with a FastDNA kit (Qbiogene, Inc., Carlsbad, CA). These served as the template for amplification of the *ergA* gene of *A. nidulans* by PCR. The DNA sequences of the PCR products were compared with that of the wild type (*pabaA1*) and also with that of the FGSC A4 strain obtained from the NCBI (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). The results showed that each of the *ergA* alleles of the TRB-resistant mutants contained a single nucleotide exchange (a T-to-C mutation at nucleotide position 1171), which led to the replacement of phenylalanine with leucine at position 391 (F391L) in ErgA from the TRB-resistant mutants (Fig. 1 and Table 1). The *ergA* nucleotide sequence of *A. nidulans* was used to BLAST search The Institute for Genomic Research *A. fumigatus* genome database for homologous sequences (http://www.tigr.org/tdb/fungal/). A nucleotide sequence with 73% sequence similarity to ErgA was obtained from the *A. fumigatus* genome database (GenBank accession no. EAL91820, AY619002.1, and AY532916) (11). The *ergA* PCR product of *A. fumigatus* was cloned into the pCR-TOPO-TA vector (Invitrogen), thus forming plasmid pRG46 (Table 1). A corresponding point mutation was then introduced into the homologous *ergA* gene at nucleotide position 1664 (T to C), resulting in the replacement of phenylalanine with leucine at position 389 (F389L). Nucleotide alterations were introduced into the 2.5-kb *ergA* gene fragment with the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA), thus forming plasmid pRG47 (Table 1). Plasmid pRG47 also contains a silent SacII site mutation within the *ergA* gene fragment to facilitate rapid identification of transformants. Transformations of *A. fumigatus* were carried out by electroporation (3) and protoplasting (5) with the plasmids listed in Table 1. For transformations carried out with plasmid pRG47, TRB-resistant colonies were directly selected upon BD Difco Antibiotic Medium 3 (AM3; Becton Dickinson & Co.) supplemented with 1.0 μg/ml TRB (lot 23925712; stock solution of 25 mg/ml; LKT Laboratories, Inc.) after 3 to 4 days of incubation at 37°C. We isolated several TRB-resistant *A. fumigatus* strains by transforming plasmid pRG47 into susceptible strain ATCC 13073 (Fig. 2). DNA analysis revealed the *ergA* gene to be ectopically integrated into the TRBR-Ec (TRB-resistant ectopic) strain, indicating that expression of the ectopic mutant gene was dominant over the wild-type allele. The selection of successful transformants was robust, suggesting potential use of the *ergA* gene as a selectable marker in *A. fumigatus*. Strain TRBR-Ec was observed to have a high level of resistance (>500-fold) to TRB in comparison with TRB-sensitive strain ATCC 13073, for which the MIC was approximately 0.3 μg/ml TRB. MICs were determined by the NCCLS M38-P microdilution method (12) in RPMI 1640 medium (Sigma Aldrich, St. Louis, MO) in the presence of 0.03 to 160.0 μg/ml TRB after 24 and 48 h of growth at 37°C. The TRBR-F389L strain did not display cross-resistance to common azole antifungal drugs such as fluconazole, clotrimazole, itraconazole, and ketoconazole (data not shown), suggesting a mechanism-specific resistance to TRB.

To determine whether the TRB resistance displayed by strain TRBR-F389L was dependent on the presence of the mutant *ergA* gene of *A. fumigatus*, we evicted plasmids pRG48 and pRG49 (Table 1) by subculturing the strain on minimal medium (MM) without drug selection. Loss of the plasmids was associated with concomitant loss of resistance to TRB on AM3 containing TRB (1.0 μg/ml) and hygromycin B (HYG; 200 μg/ml in MM; Roche Applied Science, Inc.), a selectable marker on the plasmid. Colonies that were unable to grow upon either drug-containing medium were considered to have lost the plasmid; all such strains showed fully restored susceptibility to TRB.

Thus, TRB resistance of strain TRBR-F389L was associated with the mutant *ergA* gene of *A. fumigatus*. The F389L substitution in ErgA corresponds to the F402L substitution identified in a TRB-resistant isolate of *S. cerevisiae*, located within a purported drug-binding site (8, 10). It is interesting that this phenylalanine residue is highly conserved among fungi (Fig. 1) and mammals (10). Thus, TRB resistance in strain TRBR-F389L suggests an aberrant TRB-SE interaction. The lack of cross-resistance to other antifungal classes suggested that the mechanism of resistance reflects the specific nature of drug-target interactions at the SE. In this report, we demonstrate that a single mutation in the *ergA* gene is sufficient to confer TRB resistance in *A. fumigatus*.

This work was supported by NIH grant AI066561 to D.S.P.

E.M.F.R. acknowledges Padmaja Paderu, Juan C. Robles, Rema Suresh, Guillaume Delmas, Sergey V. Balashov, Svetlana Senina, and Steven Cagas for technical support.

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