ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Nov. 2007, p. 4174–4176 Vol. 51, No. 11
0066-4804/07/$08.00+0 doi:10.1128/AAC.00917-07

A Ser678Pro Substitution in Fks1p Confers Resistance to Echinocandin Drugs in *Aspergillus fumigatus*▽

Eleusa Maria F. Rocha, Guillermo Garcia-Effron, Steven Park, and David S. Perlin*

Public Health Research Institute and the Department of Microbiology and Molecular Genetics, UMDNJ-New Jersey Medical School, Newark, New Jersey 07103-3535

Received 13 July 2007/Returned for modification 21 July 2007/Accepted 20 August 2007

An S678P substitution in Fks1p, the major subunit of glucan synthase, was sufficient to confer echinocandin resistance in *Aspergillus fumigatus*. The equivalent mutation in *Candida* spp. has been implicated in echinocandin resistance. This work demonstrates that modification of Fks1p is a conserved mechanism for echinocandin resistance in pathogenic fungi.

Echinocandin drugs are the newest class of antifungal agents available for the treatment of invasive fungal infections. They inhibit the 1,3-β-D-glucan synthase (GS), which is responsible for the synthesis of 1,3-β-D-glucan, an essential fungal cell wall component (6, 7, 8, 9, 12, 13, 14). Of the three FDA-approved echinocandin drugs, caspofungin (CSF), micafungin (MCF), and anidulafungin (ANF), only CSF has been approved for the treatment of patients with *Aspergillus* sp. infections (11). However, MCF and ANF show efficacy against invasive aspergillosis in animal models and limited patient studies (5, 22). Nevertheless, there are no documented reports of clinical failure due to echinocandin resistance, as observed with yeasts (*Candida* spp.) (10, 15, 17). In this study, modification of Fks1p was explored as a primary mechanism for echinocandin resistance in *A. fumigatus* by introduction of an S678P substitution within the major hot-spot region (21).

A point mutation into *A. fumigatus FKS1* (GenBank accession no. AFU79728) at nucleotide position 2086 (T to C), resulting in an S678P amino acid substitution, was engineered using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The equivalent mutation in the highly conserved "hot-spot 1" region of Fks1 is known to confer echinocandin resistance in *Candida* spp. (3, 20, 21) (Fig. 1). The mutant gene was cloned into the SmaI site of pRG3-(pyr4)-AMA1 (1, 2, 11), forming plasmid pRG3-AMA1-Fks1S678P (Table 1). This autonomously replicating plasmid and an HpaI-cut linearized form, which forces integration of the mutant gene into a chromosomal location, were used to transform *A. fumigatus* recipient strain KU80Δ*akuB* (4), and colonies were selected for CSF resistance on antibiotic medium 3 (AM3) supplemented with 10 μg/ml CSF. Resistant colonies that were either homozygous for the mutant *fks1* allele (EMFR-S678P), resulting from homologous recombination, or heterozygous (EMFR-S678P/WT), containing chromosomal wild-type *FKS1* and plasmid-borne mutant *fks1* (Fig. 2), were identified. Control experiments demonstrated that overexpression of wild-type *FKS1* from a plasmid was insufficient to confer resistance (not shown). DNA sequencing was used to confirm the *fks1* mutant alleles following PCR amplification with primers 5-GCAAGT GAACAATAAGCCTCCC and 5-GATGATGGCATTCCAA ACTTGAG and analysis with a CEQ 8000 capillary electrophoresis DNA sequencer (Beckman Coulter, Inc., Fullerton, CA). Representative colonies of each genetic background were evaluated for antifungal susceptibility by using CLSI reference methodology M38-A (18). Minimal effective concentrations (MEC) were determined at 24 and 48 h of growth, as described previously (14). EMFR-S678P was resistant to all three echinocandin drugs, with MEC values for each drug of ≥16 μg/ml, which contrasted with the parent wild-type strain, with MEC values of 0.015 to 0.25 μg/ml (Table 2). Similarly, EMFR-S678P/WT colonies were found to have MEC values for MCF, ANF, and CSF of 8, 8, and >16 μg/ml, respectively (Table 2).

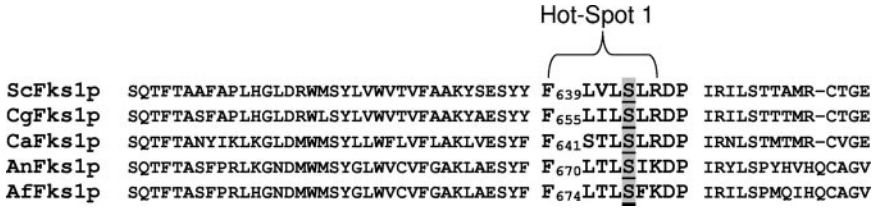

FIG. 1. Amino acid sequence alignment of Fks1p containing "hot-spot 1" regions from five fungal species: *Saccharomyces cerevisiae* (Sc), *Candida glabrata* (Cg), *Candida albicans* (Ca), *Aspergillus nidulans* (An), and *Aspergillus fumigatus* (Af). The highly conserved Ser locus is shaded.

* Corresponding author. Mailing address: PHRI, International Center for Public Health, 225 Warren St., Newark, NJ 07103. Phone: (973) 854-3200. Fax: (973) 854-3101. E-mail: perlinds@umdnj.edu.

▽ Published ahead of print on 27 August 2007.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference(s) or source
Strains		
KU80Δ	*A. fumigatus pyrGAF*::ΔKU80; wild-type strain sensitive to echinocandin drugs	4
EMFR-S678P	Homologous recombinant *fks1*(*S678F*) mutant of KU80 formed following transformation with linear pRG3-(pyr4)-AMA1-Fks1S678P cut with HpaI	This study
EMFR-S678P/WT	*fks1* mutant of KU80 transformed with uncut autonomously replicating plasmid pRG3-(pyr4)-AMA1-Fks1S678P; also contains wild-type *FKS1*	This study
EMFR-FKS1-Restored	Strain derived from EMFR-S678P/WT after plasmid eviction	This study
Plasmids		
pRG3-AMA1	Contains *A. nidulans AMA1* and *pyr4* genes	1, 2, 16
pRG3-AMA1-Fks1S678P	Contains *A. fumigatus fks1*(*S678P*) in pRG3-(pyr4)-AMA1	This study

The observed resistance phenotype of EMFR-S678P/WT suggested that expression of the mutant allele from a plasmid in a wild-type background was genetically dominant. This property was used to further validate the role of Fks1p modification in resistance. Four independent colonies of EMFR-S678P/WT were serially subcultured onto AM3 without drug. After serial passages ($n = 10$), loss of plasmid was determined by inoculating cells onto AM3 containing CSF (10 μg/ml). Colonies unable to grow on media containing drug were considered to have lost the plasmid. These colonies were then retested for their susceptibility to echinocandin drugs, and all were found to have completely restored susceptibility to drug (Fig. 2B). Neither plasmid nor the mutant *fks1* allele could be detected in these cells by PCR amplification, which confirmed the loss of the plasmid and validated the role of the mutant *fks1* gene in conferring resistance.

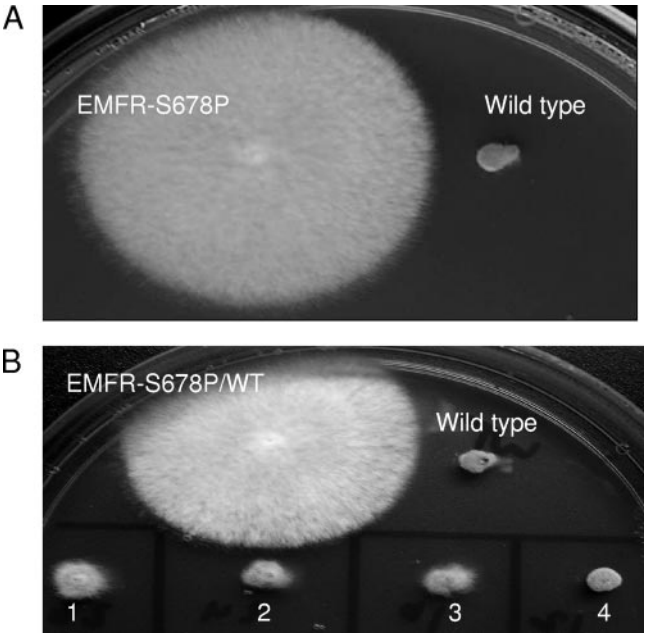

FIG. 2. CSF-sensitive and -resistant isolates of *A. fumigatus*. (A) Growth of strains EMFR-S678P (mutant) and KU80Δ (wild type) on AM3 with 10.0 μg/ml of CSF plus pyrimidine. (B) Growth of EMFR-S678P/WT containing plasmid-expressed *fks1*(*S678P*) (top set). EMFR-FKS1-Restored isolates 1 to 4 with restored sensitivity to echinocandin following loss of plasmid (bottom set). Strains were grown for 72 h at 37°C on AM3 with 10.0 μg/ml of CSF.

In *Candida albicans*, *fks1* mutations are manifested as decreased sensitivity of GS to echinocandin drugs (20, 21). To assess echinocandin sensitivity of GS from wild-type and *fks1*(*S678P*) mutant strains of *A. fumigatus* (19), product-entrapped enzymes were isolated; the kinetic inhibition profiles are shown in Fig. 3. The introduction of *fks1*(*S678P*) caused a pronounced decrease in the sensitivity of GS to all three drugs, resulting in 50% inhibitory concentration (IC_{50}) values increased 121-, 122-, and 20-fold for CSF, ANF, and MCF, respectively (Table 2). This characteristic IC_{50} shift in enzyme sensitivity was consistent with the drug-resistant phenotypes observed (Table 2) and is in accord with similar behavior with other fungi (21). The kinetic properties of *fks1*(*S678P*) expressed from plasmid (strain EMFR-S678P/WT) were more complex, displaying dual IC_{50} values due to the mixed enzyme species present (Fig. 3D). However, once the plasmid was lost (EMFR-FKS1-Restored), GS showed complete restoration of enzyme sensitivity (IC_{50} of <0.9 ng/ml) to all echinocandin drugs (Fig. 3A to C).

These data provide evidence that modification of Fks1p in *A. fumigatus* is necessary and sufficient to confer resistance to echinocandin drugs. Given the emerging importance of this conserved mechanism for clinical resistance in pathogenic fungi, it would be prudent to be alert to the potential evolution of this mechanism for echinocandin drugs in *Aspergillus* spp., especially as echinocandin mold therapy expands.

TABLE 2. Echinocandin inhibition of growth and GS with wild-type and mutant strains

Strain	MEC (μg/ml)[a]			IC_{50} (ng/ml)[b]		
	CSF	ANF	MCF	CSF	ANF	MCF
KU80Δ	0.25	0.015	0.015	0.91	0.24	0.63
EMFR-S678P	>16	16	>16	109.70	29.17	12.61
EMFR-S678P/WT	>16	8	8	0.12, 104.9	0.37, 29.89	0.50, 14.38

[a] Geometric means (three repetitions from separate preparations) are given.
[b] Multiple IC_{50} values reflect kinetic parameters for mixed wild-type and mutant enzyme populations.

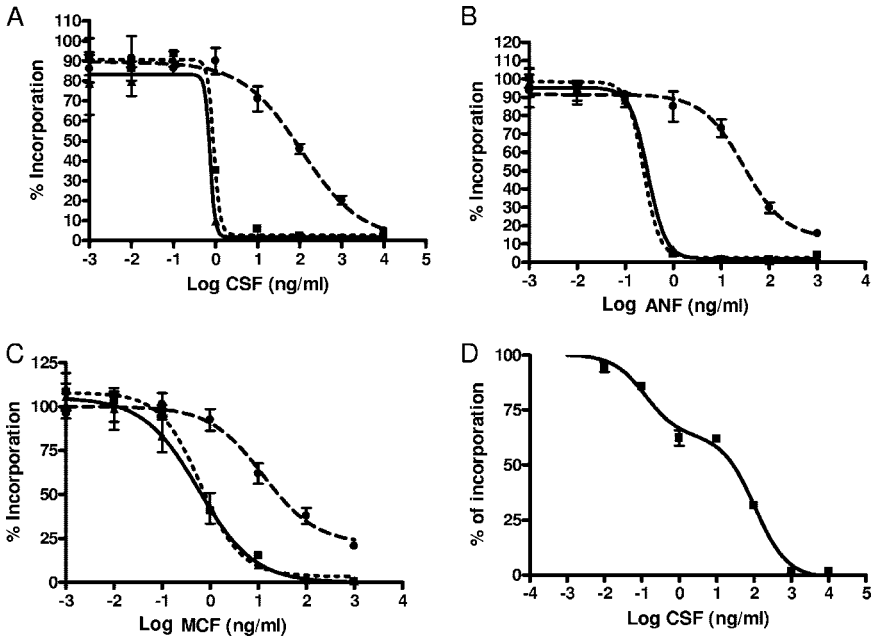

FIG. 3. Echinocandin inhibition profiles of enriched GS complexes from the wild type (triangles), EMFR-S678P (circles), and EMFR-FKS1-Restored (squares). The enzymes were evaluated with CSF (A), ANF (B), and MCF (C). The mixed inhibition profile to CSF for GS from EMFR-S678P/WT is also shown (D). Relative GS activities shown in all panels were assessed by the incorporation of [^{3}H]glucose into the radiolabeled product.

This work was supported by NIH grants AI066561 and AI069397 to D.S.P.

We thank Rebecca Gardiner for helpful discussions and Jean-Paul Latgé (Institut Pasteur, France) and Gustavo H. Goldman (Universidade de Sao Paulo, Brazil) for providing the KU80Δ *A. fumigatus* strain.

REFERENCES

1. **Aleksenko, A., and A. J. Clutterbuck.** 1996. The plasmid replicator AMA1 in *Aspergillus nidulans* is an inverted duplication of a low-copy-number dispersed genomic repeat. J. Mol. Microbiol. **19:**565–574.
2. **Aleksenko, A., and A. J. Clutterbuck.** 1997. Autonomous plasmid replication in *Aspergillus nidulans AMA1* and *MATE* elements. Fungal Genet. Biol. **21:**373–387.
3. **Balashov, S. V., S. Park, and D. S. Perlin.** 2006. Assessing resistance to the echinocandin antifungal drug caspofungin in *Candida albicans* by profiling mutations in *FKS1*. Antimicrob. Agents Chemother. **50:**2058–2063.
4. **da Silva Ferreira, M. E., M. R. V. Z. Kress, M. Savoldi, M. H. S. Goldman, A. Härtl, T. Heinekamp, A. A. Brakhage, and G. H. Goldman.** 2006. The *akuB*^{KU80} mutant deficient for nonhomologous end joining is a powerful tool for analyzing pathogenicity in *Aspergillus fumigatus*. Eukaryot. Cell **5:**207–211.
5. **Denning, D. W.** 2006. Aspergillus and aspergillosis—progress on many fronts. Med. Mycol. **44**(Suppl.)**:**1.
6. **Denning, D. W.** 2002. Echinocandins: a new class of antifungal. J. Antimicrob. Chemother. **49:**889–891.
7. **Douglas, C. M., F. Foor, J. A. Marrinan, N. Morin, J. B. Nelsen, A. M. Dahl, P. Mazur, W. Baginasky, W. Li, M. El-Sherbeini, J. A. Clemas, S. M. Mandala, B. R. Frommer, and M. B. Kurtz.** 1994. The *Saccharomyces cerevisiae FKS1* (*ETG1*) gene encodes an integral membrane protein which is a subunit of 1,3-β-D-glucan synthase. Proc. Natl. Acad. Sci. USA **91:**12907–12911.
8. **Douglas, C. M., J. A. Marrinan, W. Li, and M. B. Kurtz.** 1994. A *Saccharomyces cerevisiae* mutant with echinocandin-resistant 1,3-β-D-glucan synthase activity. J. Bacteriol. **176:**5686–5696.
9. **Douglas, C. M., J. A. D'Ippolito, G. J. Shei, M. Meinz, J. Onishi, J. A. Marrinan, W. Li, G. K. Abruzzo, A. Flattery, K. Bartizal, A. Mitchell, and M. B. Kurtz.** 1997. Identification of the *FKS1* gene of *Candida albicans* as the essential target of 1,3-β-D-glucan synthase inhibitors. Antimicrob. Agents Chemother. **41:**2471–2479.
10. **Hakki, M., J. F. Staab, and K. A. Marr.** 2006. Emergence of a *Candida krusei* isolate with reduced susceptibility to caspofungin during therapy. Antimicrob. Agents Chemother. **50:**2522–2524.
11. **Hope, W. W., S. Shoham, and T. J. Walsh.** 2007. The pharmacology and clinical use of caspofungin. Exp. Opin. Drug Metab. Toxicol. **3:**263–274.
12. **Kurtz, M. B., G. Abruzzo, A. Flattery, K. Bartizal, J. A. Marrinan, W. Li, J. Milligan, K. Nollstadt, and C. M. Douglas.** 1996. Characterization of echinocandin-resistant mutants of *Candida albicans*: genetic, biochemical, and virulence studies. Infect. Immun. **64:**3244–3251.
13. **Kurtz, M. B., and C. M. Douglas.** 1997. Lipopeptide inhibitors of fungal glucan synthase. J. Med. Vet. Mycol. **35:**79–86.
14. **Kurtz, M. B., I. B. Heath, J. Marrinan, S. Dreikorns, J. Onishi, and C. M. Douglas.** 1994. Morphological effects of lipopeptides against *Aspergillus fumigatus* correlate with activities against (1,3)-β-D-glucan synthase. Antimicrob. Agents Chemother. **38:**1480–1489.
15. **Laverdiere, M., R. G. Lalonde, J. G. Baril, D. C. Sheppard, S. Park, and D. S. Perlin.** 2006. Progressive loss of echinocandin activity following prolonged use for treatment of *Candida albicans* oesophagitis. J. Antimicrob. Chemother. **57:**705–708.
16. **Liu, W., G. S. May, M. S. Lionakis, E. R. Lewis, and P. Kontoyiannis.** 2004. Extra copies of the *Aspergillus fumigatus* squalene epoxidase gene confer resistance to terbinafine: genetic approach to studying gene dose-dependent resistance to antifungals in *A. fumigatus*. Antimicrob. Agents Chemother. **48:**2490–2496.
17. **Miller, C. D., B. W. Lomaestro, S. Park, and D. S. Perlin.** 2006. Progressive esophagitis caused by *Candida albicans* with reduced susceptibility to caspofungin. Pharmacotherapy **26:**877–880.
18. **National Committee for Clinical Laboratory Standards.** 2002. Reference method for broth dilution antifungal susceptibility testing of filamentous fungi. Approved standard M38-A. National Committee for Clinical Laboratory Standards, Wayne, PA.
19. **Paderu, P., G. Garcia-Effron, S. Balashov, G. Delmas, S. Park, and D. S. Perlin.** 2007. Serum differentially alters the antifungal properties echinocandin drugs. Antimicrob. Agents Chemother. **51:**2253–2256.
20. **Park, S., R. Kelly, J. N. Kahn, J. Robles, M. J. Hsu, E. Register, W. Li, V. Vyas, H. Fan, G. Abruzzo, A. Flattery, C. Gill, G. Chrebet, S. A. Parent, M. Kurtz, H. Teppler, C. M. Douglas, and D. S. Perlin.** 2005. Specific substitutions in the echinocandin target Fks1p account for reduced susceptibility of rare laboratory and clinical *Candida* sp. isolates. Antimicrob. Agents Chemother. **49:**3264–3273.
21. **Perlin, D. S.** 2007. Resistance to echinocandin-class antifungal drugs. Drug Res. Updates **10:**121–130.
22. **Vehreschild, J. J., T. Kummerle, M. Karthaus, and O. A. Cornely.** 2007. Anidulafungin—state of affairs from a clinical perspective. Mycoses **50:**38–43.