Medical Mycology October 2012, **50,** 716–725

informa
healthcare

β-(1,3)-glucan synthase complex from *Alternaria infectoria*, a rare dematiaceous human pathogen

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The fungal cell wall polymer β-(1,3)-D-glucan is synthesized by the enzyme β-(1,3)-D-glucan synthase that is a complex composed of at least two proteins, Rho1p and Fks1p. Here, we report the nucleotide sequence of a single *FKS* gene and of the regulatory unit, *RHO1* from the dematiaceous pathogenic fungus *Alternaria infectoria*. The predicted AiFks and AiRho share, respectively, 93% and 100% identity with that of *Drechslera tritici-repentis*. We also report that the sensitivity to caspofungin of eight different *A. infectoria* clinical strains is similar, with a MIC $>$ 32 μg/ml and a MEC of 1 μg/ml, except for one strain which had a MEC of 1.4 μg/ml. This same strain exhibited one substitution at the hot spot 2, S1405A, compatible with less susceptible phenotypes, with the other seven strains having no mutations in either hot spot 1 or 2. The relative quantification of the expression of *AiFKS* and of *AiRHO* demonstrated a decrease in response to an exposure to caspofungin at 0.5 μg/ml.

Keywords β-(1,3)-glucan synthase, *FKS* gene, *Alternaria infectoria*, caspofungin

Introduction

Filamentous fungi with cell walls containing melanin belong to the order *Pleosporales*, their anamorphs classically referred as members of the *Dematiaceae* [1]. These are ubiquitous environmental fungi, occurring in plants, soil, food and indoor air environments, and as agents of human infection, phaeohyphomycosis, usually affecting the sub-cutaneous tissue [2] and, in particular, the central nervous system [3]. Among the *Dematiaceae*, *Alternaria* species are increasingly found as aetiologic agents of human disease, due to the growing number of immunocompromised patients [1]. *Alternaria infectoria* is a rare opportunistic agent of phaeohyphomycosis [4] and a PubMed search revealed several human clinical cases, some of which involve deep organic infections [5].

Caspofungin, an antifungal belonging to the class of echinocandins, has been widely used in human mycoses due to its high efficiency and low number of side effects [6,7]. Caspofungin is a non-competitive inhibitor of the β-(1,3)-D–glucan synthase, meaning that this drug target is the fungal cell wall synthesis, considered to be an ideal target for antifungal drugs. The gene coding for this enzyme, the *FKS* gene, was first identified in *Saccharomyces cerevisiae* as a gene confering hypersensitivity to FK506 and cyclosporin A [8]. In *S. cerevisiae* there are two FKS genes, *FKS1* and *FKS2* [9], both coding for catalytic subunits and regulated by Rhop that in turn interacts with Pkc1p [10,11]. The fact that the protein coded by the *FKS* gene is the target for echinocandins has led to a thorough effort to identify the gene(s) responsible for this cell wall synthesis enzyme in human fungal pathogens [12–15]. *Candida albicans*, the most frequent human pathogenic fungi, has three *FKS* genes [16].

The *in vitro* potency of caspofungin and of the other echinocandins against clinical isolates has been thoroughly documented. Nevertheless, as episodes of clinical failures began to be reported [5,17], the existence of mutations linked to reduced susceptibility to echinocandins were identified in *Candida* spp. [18,19] and in the filamentous fungi *Fusarium solani*, *Scedosporium prolificans* and *Aspergillus fumigatus* [20,21]. These mutations are mostly

Received 26 September 2011; Received in final revised form 9 January 2012; Accepted 10 March 2012.

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DOI: 10.3109/13693786.2012.675525

located in the two highly conserved hot spot regions1 and 2 [19,22]. Some point mutations are considered to be either intrinsic like in the *Candida parapsilosis* family or in *Candida guilliermondi* (for a review see ref. 17), or induced during breakthrough of echinocandin treatment [20,23].

A case of cerebral phaeohyphomycosis due to *A. infectoria* in which caspofungin was used as the therapeutic approach with an initial period of remission of the fungal abscess followed by a period of inefficient control of fungal development [5], prompted us to identify in this dematiaceous fungus, not only the *FKS* gene but also its regulatory unit, *RHO1*. The transcriptional level of the two identified genes in fungal cells exposed to caspofungin was measured using real-time RT-PCR. The susceptibility to caspofungin was quantified *in vitro*, comparing several clinical isolates of this fungal species. The recognition of the linkage between caspofungin susceptibility and genotype lead us to study, in *A. infectoria*, the existence of point mutations in the hot spot regions described for other fungi.

Materials and methods

Organisms and media

A total of eight *A. infectoria* clinical isolates were used in this study. One was recovered in our laboratory (IMF001; deposited at CBS as CBS 122351) and the other seven were obtained from CBS (Table 1). The strains were stored at − 80°C.

Preparation of inocula and caspofungin susceptibility testing

Since seven of the tested *A. infectoria* strains failed to produce spores, the inocula suspensions for susceptibility assays were prepared using fragmented hyphae [24]. Fungi were grown in liquid medium for 5 days, and the mycelium obtained was homogenized in a MagNA Lyser (Roche) under mild conditions (6500 rpm, 25 s). The homogenate was centrifuged at low speed to remove the larger hyphal fragments and the supernatant containing the smaller fragments was used as inocula. The viability and density of the inoculum was tested by spreading portions onto PDA media and colony counts.

Caspofungin (CAS; Merck & Co, Inc., Rahway, NJ) was obtained as a standard powder (caspofungin acetate) and dissolved in sterile distilled water. Microdilution broth assays were determined using the M38-A method from the CLSI, with minor changes. The results were evaluated after incubation at 30°C for 48 h, except for some slow growing strains (IMF010 and IMF011) that required 72 h of incubation. The lowest concentration producing significant macroscopic changes in hyphal morphology was considered the end point in assessing the minimum effective concentration (MEC). Some of the tested strains grew well at 30°C but poorly at 35°C and for them the microdilution broth assays readings were only possible after 96 h.

Radial growth inhibition and β-(1,3)-D-glucan assays were performed as previously described [25]. Depending on the strain, hyphal fragments or conidia ($1–5 \times 10^6$/ml) were applied at a single point on the surface of plates of potato dextrose agar (PDA Difco) containing 1 μg/ml CAS. Plates were incubated at 30°C for 5 days under 12 h-alternating light (lamp F15W T8BLB) and dark cycle. Radial diameter of growth was measured and compared relative to control plates lacking caspofungin.

FUN-1 staining and microscopy

A. infectoria conidia, harvested from 1–2 weeks old PDA cultures, were incubated, as described above, on antibiotic medium 3 (AM_3; Difco) with 0.1 % agar in the absence or presence of 5 μg/ml of CAS. Germlings were stained for 30 min at 30°C with FUN-1 (F-7030; Invitrogen) diluted to 20 μM in 20 mM HEPES buffer with 2% glucose, pH 7.2. Microscopy was performed using an Olympus BX-40 microscope (equipped for fluorescence with a fluorescein isothiocyanate filter) at 400 × total magnification. Images

Table 1 Strains of *Alternaria infectoria* used in this study.

Strain		Clinical setting[1]
IMF001	CBS 122351	Brain abscess CGD patient[2]
IMF006	CBS 137.90	Granulomatous lesion on arm
IMF007	CBS 102692	Cutaneous lesions, heart transplant recipient
IMF008	CBS 109785	Skin lesion of male transplant recipient
IMF009	CBS 110803	Skin lesion arm
IMF010	CBS 110804	Skin
IMF011	CBS 115832	Cutaneous infection of 53-year-old man under prednisolone therapy
IMF012	CBS 117210	Skin lesion

[1]The clinical setting of each of the *Alternaria infectoria* here indicated is that available at the CBS data base (www.cbs.knaw.nl)

[2]Ref. 5

were recorded at different time periods on an Olympus C-200 digital camera.

Identification and sequencing of FKS *and* RHO1

For RNA and DNA isolation, liquid cultures of *A. infectoria* (strain IMF001) were harvested by centrifugation and immediately frozen in liquid nitrogen. Cell extracts were prepared by grinding the frozen samples with a mortar and pestle. Total RNA was isolated using TRI REAGENT (Sigma Aldrich) according to the manufacturer's instructions. Genomic DNA isolation was performed as described previously [26]. Reverse transcription of 3 μg of total RNA was performed using the 1st Strand cDNA synthesis kit for RT-PCR (Roche) according to the manufacturer's instructions.

For the *A. infectoria FKS1* gene identification, genomic DNA was used for PCR amplification with the degenerate primers FKSfor3 and FKSrev1 (Table 2), derived from highly conserved regions of other fungal Fks1 proteins. The gene specific primers FKSfor4 and FKSrev5 were designed based on the sequence amplified with the first pair of primers and used to amplify the 5′ and the 3′ ends of cDNA by RACE-PCR with FirstCHoice RLM-RACE kit (Ambion). The complete FKS1 genomic DNA sequence was amplified using primers FKSfor24 and FKSrev11 (Table 2). The PCR amplified fragments were cloned into pcrSMART vector (Lucigen Corporation) and sequenced elsewhere.

Table 2 Nucleotide sequence of the primers used in this study.

Name	Sequence (5′ → 3′)
FKSfor3	GAYGCBAAYCARGAYAAYTA
FKSrev1	ACCYTTNCCRCAYTGRWARTA
FKSfor4	TCTCAACGAGGATATTTACGCTGGTATGA
FKSrev5	CTGCGAATCTTCAGGCACTCTT
FKSfor24	GGCAGCACGACTACGAGCAAT
FKSrev11	AACGAAGGAAATGCTGTAGTGGATA
FKSfor14	GTTCGTTGATGATGCTGCTG
FKSrev10	AAGATGTTCGTGAAGTGAGC
18Sfor	CGGCTACCACATCCAAGGAA
18Srev	GCTGGAATTACCGCGGCT
RHOfor3	TCGACAACGTCCAGGAGA
RHOrev2	ATACTTCTCGGACGCCCTC
RHO1for2	TGGGATACBGCTGGNCARGARGAYTA
RHO1rev1	TCYTGWCCRGCRGTATCCCA
FKS1800f	GTCTACATTCTTGGTATGGA
FKS2500r	GAACCTGGTGGTAAAGCAAC
FKS1850f	ACTATCGCCAACGTTCTCGGTGGT
FKS2600r	AGCGAAGAAAGAGATACGGCGTT
FKS3900f	TTTGGTTCCGTCCTCAACTT
FKS4500r	GTAGATGGAAGGACCGGCGA
FKSd1f	AAYCAIGAYAAITAIYTIGA
FKSd1r	TTICCRCAITGITAITAYTC
Fksd2f	CAYGCNGAYTAYATHGGNGGNGA
Fksd2r	ACYTGRTTNGCYTCNCCCCARCA

A similar strategy was used for cloning *A. infectoria RHO1* gene. In this case the degenerate primers used were RHO1for2 and RHO1rev1 and the specific primers were RHOfor3 and RHOrev2 (Table 2).

Search for other hypothetical FKS isoforms and determination of the amino acid sequence of the two hot spots of the eight A. infectoria clinical isolates used in this study

Degenerate primers (FKSd1f, FKSd1r and Fksd2f, Fksd2r; Table 2) were tested as a means of discarding the existence of more than one FKS gene. PCRs were performed at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, 48°C for 1 min and 72°C for 1 min, an additional extension time of 7 min at 72°C were performed. PCR products were visualized in a 1% agarose gel. PCR products of the expected size were extracted from the gel and purified to be further sequenced. For unsuccessful amplifications, we attempted a lower stringency PCR which included one cycle at 94°C for 3 min; 10 cycles of 92°C for 30 s, 45°C for 1 min and 72°C for 1 min; 30 cycles at 92°C for 30 s, 40°C for 1 min and 72°C for 1 min; 1 cycle at 72°C for 5 min [27].

Primers FKS1800f and FKS2500r were designed to amplify hot spot 1 of the eight *A. infectoria* clinical isolates but we failed to amplify the hot spot 1 from the IMF006 strain. To overcome this situation, we designed another pair of primers, i.e., FKS1850f and FKS2600r (Table 2). Hot spot 2 was amplified using the primers FKS3900f and FKS4500r (Table 2). These amplifications were carried out at 94°C for 5 min, followed by 35 cycles at 94°C for 1min, 50°C for 1 min and 72°C for 1 min, an additional extension time of 7 min at 72°C were performed. PCR products were visualized in a 1% agarose gel. PCR products of the expected size were isolated from the gel and purified to be further sequenced. The sequences obtained by sequencing were aligned with ClustalW (www.ebi.ac.uk).

Relative quantification of gene expression

A. infectoria (IMF001) liquid cultures, cultivated during 3 days on AM3, were incubated in the presence of different concentrations of CAS (0, 0.5 and 5 μg/ml). After 7 h, the hyphae were collected by centrifugation and frozen in liquid nitrogen. Total RNA and cDNA synthesis from each sample was performed as described above. The relative quantification of *FKS1* and *RHO1* gene expression was performed using the 18S ribosomal RNA as the reference gene. The real-time PCR reactions were performed in a LightCycler 2.0 (Roche Diagnostics), using a LightCycler II Fast Start DNA MasterPlus SYBR Green I kit (Roche Diagnostics). The primers used in the real-time LC protocols were for *FKS*, FKSfor14 and FKSrev10, for *RHO*, RHOfor3 and RHOrev2, and for *18S*, 18Sfor and 18Srev

(Table 2). The expression values were normalized to the values for the reference gene using the method previously described [28].

Results

Susceptibility to caspofungin

While eight clinical *A. infectoria* strains were used in this study, seven did not form conidia or did so poorly that inocula suspensions for susceptibility assays were prepared using fragmented hyphae. The described inocula preparation methodology proved to be efficient since we were able to obtain reproducible results whenever the same concentration of hyphal fragments was used, as determined by CFU counts. All the *in vitro* antifungal assay methods used to assess susceptibility to caspofungin (radial growth inhibition, microdilution assay and β-(1,3-D)-glucan assay) indicated that *A. infectoria* growth was inhibited by CAS. Nevertheless, caspofungin, even at the highest tested concentration (32 μg/ml), did not fully prevent *in vitro* growth of *A. infectoria*. However, CAS induced abnormal hyphal growth with short abundant branching (Fig. 1), revealing similar MECs (1 μg/ml) in the group of tested strains. However, one of the strains, IMF006, revealed a slightly higher MEC value (1.4 μg/ml; geometric mean) than the other isolates. In order to assess the presence in *A. infectoria* of a probable paradoxical effect, i.e., the reported tendency of caspofungin to be less inhibitory at high concentrations, we employed a concentration of 10 μg/ml to study the radial growth effect. In none of the strains did we observe an increased growth in this concentration of CAS (results not shown).

Fungal viability and morphology

The role of CAS on the viability and morphology of *A. infectoria* was assessed with conidia exposed to FUN-1, a membrane-permeant probe that is freely taken up and converted from a diffusely distributed pool of yellow-green fluorescent intracellular stain into compact red-orange fluorescent intravacuolar structures. This conversion requires both plasma membrane integrity and metabolic activity. Only metabolically active cells show fluorescent intravacuolar structures, while dead cells exhibit extremely bright, diffuse, yellow-green fluorescence [29].

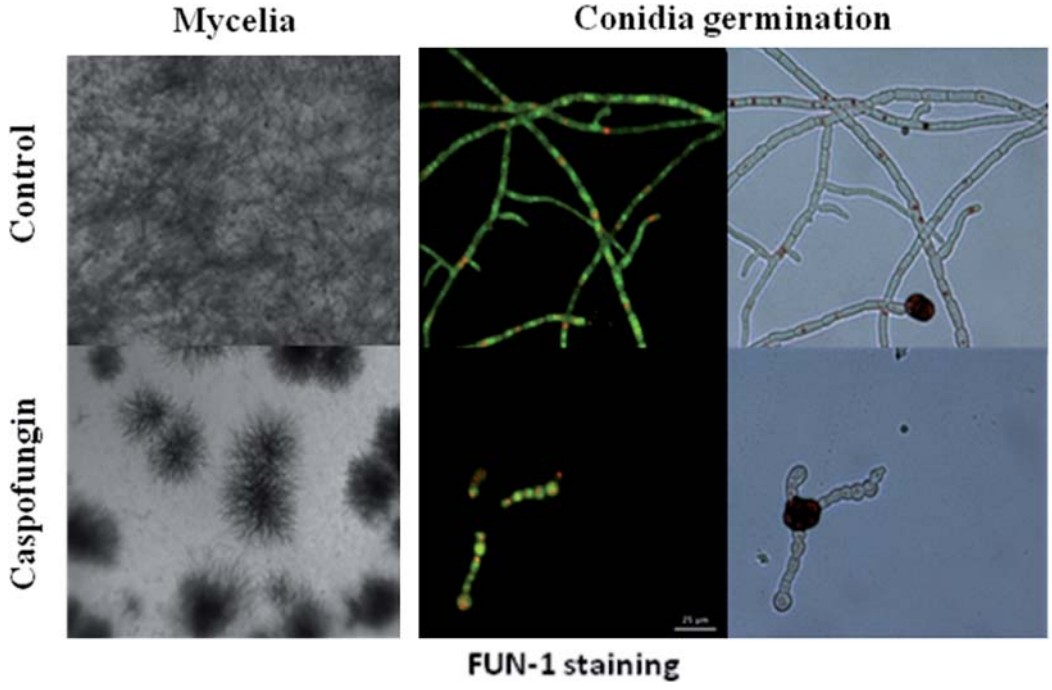

Fig. 1 Morphological change of *Alternaria infectoria* upon caspofungin exposure. In the left panels *A. infectoria* (IMF001) was grown as indicated for the microdilution broth assays, according to the M38-A method from the CLSI, in RPMI-1640 with or without CAS 16 μg/ml (representative photo). Images were taken under a Zeiss Stemi DV 4 Stereomicroscope. The remaining panels show the morphological change of *A. infectoria* conidia germination in the presence of caspofungin. *A. infectoria* conidia were loaded with FUN-1 20 mM probe for 30 min at 30°C as described under Materials and methods. The microscopic images were obtained in an Olympus BX-40 microscope, either by light microscopy or by fluorescence microscopy (right panels) after 30 h of incubation with CAS 5 μg/ml at 30°C. Images were recorded in an Olympus C-200 digital camera with a magnification of 400×.

Fluorescence microscopy revealed profound morphological changes when mycelia were incubated for 30 h with CAS at 5 μg/ml (Fig. 1). There were very short fragments, a higher degree of branching, and round globoid tips, although the hyhae were metabolically active (Fig. 1, FUN-1 staining). These morphological features contrasted with the normal long, septate hyphae in control cultures. The bursting of the hyphal tips, observed in *A. fumigatus* [30] was never observed, even at the highest concentrations tested.

Caspofungin effect in glucan content

The quantitative cell wall β-(1,3-D)-glucan measurement revealed a similar caspofungin effect within the concentration range tested (Fig. 2), when assayed using the aniline blue method as described earlier [25]. An average IC_{50} of 0.25 μg/ml was measured.

Isolation and characterization of A. infectoria FKS1 *and* RHO1 *genes*

For the cloning of the *FKS1* and *RHO1* genes from *A. infectoria*, a specific product was obtained by PCR amplification from genomic DNA using degenerate primers derived from conserved sequences in all the fungal FKS. Specific primers were further designed from the sequenced fragment and the 5′and 3′ termini from the cDNA were obtained by RACE, as described above.

The genomic DNA sequence of the *FKS1* ORF of *A. infectoria* of 5,955 bp long was deposited in the GenBank database under Accession No JF742672. This ORF was separated into three exons by two introns located at the N terminus (nt 353 to 404) and at the C terminus (nt 5721 to 5769).

A particular ATG was selected as the initiator based on the absence of other downstream or upstream possible ATGs in frame and on homology of the coded sequence with other FKS proteins. The sequence, that we designated as AiFks1 contains 1,951 amino acids with 93% identity with *FKS* of *D. tritici-repentis*. AiFks1 also displayed a high degree of similarity to the FKS homologues from *Phaeosphaeria nodorum* (90%), *Exophiala dermatitidis* (80%), *Coccidioides posadasii* (79%) and *A. fumigatus* (77%) (Fig. 3A). The sequence encodes a predicted protein with a molecular mass of 223.06 kDa and a pI of 8.21 (Proteomics ExPASy Server). A search using the InterProScan, from EMBL-EBI, identified amino acids 860 to 1691 belonging to a conserved domain of β-(1,3)-glucan synthases.

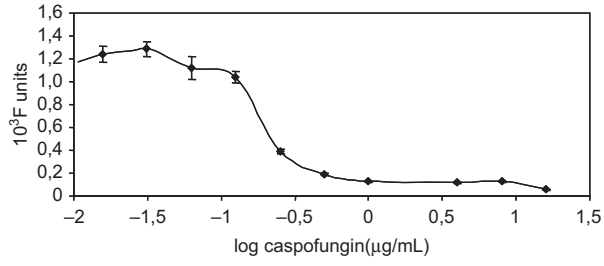

Fig. 2 β-1,3-D-glucan decrease in *Alternaria infectoria* cells incubated with CAS (0.0625–5 μg/ml) . Glucan was assessed using the aniline blue assay fluorescence in the mycelium of *A. infectoria* grown in the presence of different concentrations of caspofungin (representative graph).

Hydropathy analysis by the Top Pred2 program [31] predicted the AiFks1 as an integral membrane protein displaying about 16 transmembrane helices. The topology analysis (Fig. 4) was similar to other glucan synthases, with a large hydrophilic cytoplasmic domain of 599 amino acids [14,32].

Independent amplification of genomic DNA and cDNA with the same set of 5′ and 3′ specific terminal primers yielded the complete sequence of *RHO1* gene and cDNA from *A. infectoria*. A DNA fragment of 1321 base pairs was cloned. The analyses of this fragment returned an ORF of 582 bp long that was interrupted by four introns spanning nucleotides 139–206, 233–357, 558–606, 742–791. This gene, that we named Ai*RHO1* encoded a predicted protein of 193 amino acids with an estimated molecular size of 21.8 kDa and a pI of 6.23. The amino acid sequence of AiRHO1 was identical to the Rho homologues from *D. tritici-repentis* (100%) and shares high identity with its homologues from *Blumeria graminis* (93%), *Magnaporthe grisea* (92%) and *Aspergillus niger* (92%) (Fig. 3B). AiRho1 has GTP binding and hydrolysis consensus sequences identical to those of yeast Rho proteins. The complete Ai*RHO1* gene was deposited in the GenBank database under Accession No. JF742673.

The intron sequences of both these genes are flanked by 5′ GT and 3′ AG, which correspond to the consensus sequence of known splicing sites.

Search for other FKS *in* A. infectoria *and hot spot mutations*

A degenerate PCR strategy was utilized to assess the existence of other(s) FKS gene(s). For that, we performed PCR at low stringency [27] with the degenerate primers FKSd1f and FKSd1r, from the conserved regions of amino acid sequences from the *FKS* genes of *A. fumigatus*, *C. albicans*, *Cryptococcus neoformans*, and *S. cerevisiae*. This approach was also attempted with Fksd2f and Fksd2r degenerate primers designed after conserved amino acid sequences found in β-(1,3)-glucan catalytic subunits from *A. nidulans* (*fksA*) and *S. cerevisiae* (*FKS1*/*FKS2*) [33]. Only the pair of primers Fksd2f/Fksd2r enabled the amplification of a band with approximately 320 bp that, after

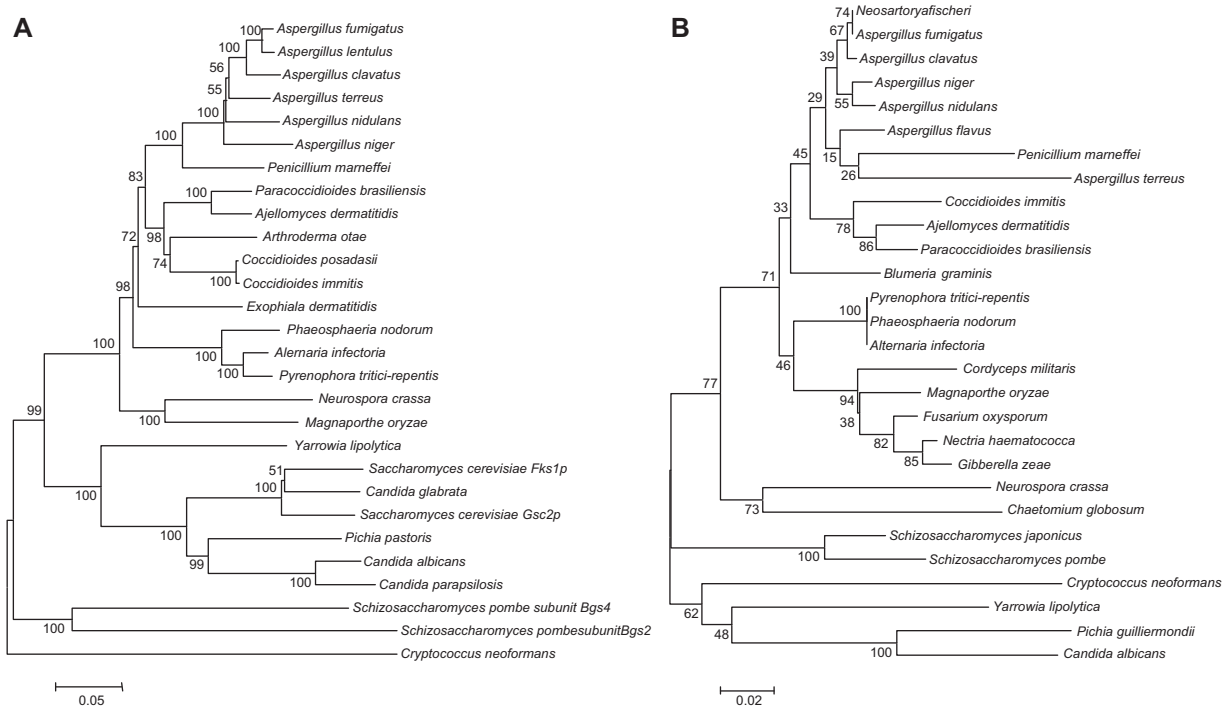

Fig. 3 Unrooted phylogenetic trees based on available amino acid sequences of identified and putative FKS (A) and e RHO (B). The Clustal X program [41] was used for sequence alignment, and the MEGA4 program [42] was used to generate the phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method [43]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The bootstrap values are shown at the nodes. Bar, 0.05 or 0.02 change/site.

sequencing, proved to have total homology with *FKS1*. Southern blot analysis provided no conclusive evidence of multiple *FKS* copies (results not shown). Therefore all molecular analysis indicated the existence of a single copy of *FKS1*.

Previous results indicate that single-amino acid substitution located in the highly conserved hot spot 1 region within the consensus Fks1p sequence are sufficient to confer reduced susceptibility to echinocandins in *S. cerevisiae* and in the pathogens *C. albicans* and *C. krusei* [20,22,34]. The analysis of this eight-amino acid region of AiFks of all eight clinical strains tested (F_{691}LTLSIKD) did not reveal any mutations that have been mapped to confer reduced echinocandin susceptibility. The same sequence is found in the key amino acid residues from *A. nidulans*. The AiFks hot spot 2 amino acids sequence of seven of the eight strains tested were W_{1403}VRRCIVS. The IMF006 strain exhibits one substitution: S1405A, compatible to a less susceptible phenotype (Fig. 4A).

Relative expression of AiFKS *and* AiRHO *genes changes with CAS exposure*

Sybr Green real time RT-PCR was used to evaluate the relative gene expression of AiFKS and AiRHO. This assessment of the transcriptional level of both genes was performed in fungal cells (*A. infectoria* IMF001) exposed to 0.5 and to 5 μg/ml of CAS for 7 h. The idea was to measure the relative expression of both genes in fungal cells growing in different concentrations of CAS, one of which is sub-inhibitory, 0.5 μg/ml, and another confirmed as inhibiting the *A. infectoria* growth and the total content of β-glucan, 5 μg/ml. The reference gene used was the 18S, and the values were normalized to the levels of expression of this gene [28]. The results obtained show that when compared to the control cells, fungal cells exposed to CAS 0.5 μg/ml for 7 h, show a decrease in the expression of both *AiFKS* and *AiRHO* of 50% (Fig. 5). The transcriptional level of both genes increased to 80% when the fungal cells were grown in a higher concentration of CAS, 5 μg/ml. These results express a single time point of exposure to caspofungin (7 h) and were generated from two independent biological assays.

Discussion

Phaeohyphomycotic fungi arose lately as a group which have increasingly been found as human pathogens [35]. They cause central nervous system infections, which are difficult to diagnose and treat [3]. *A. infectoria* is one of

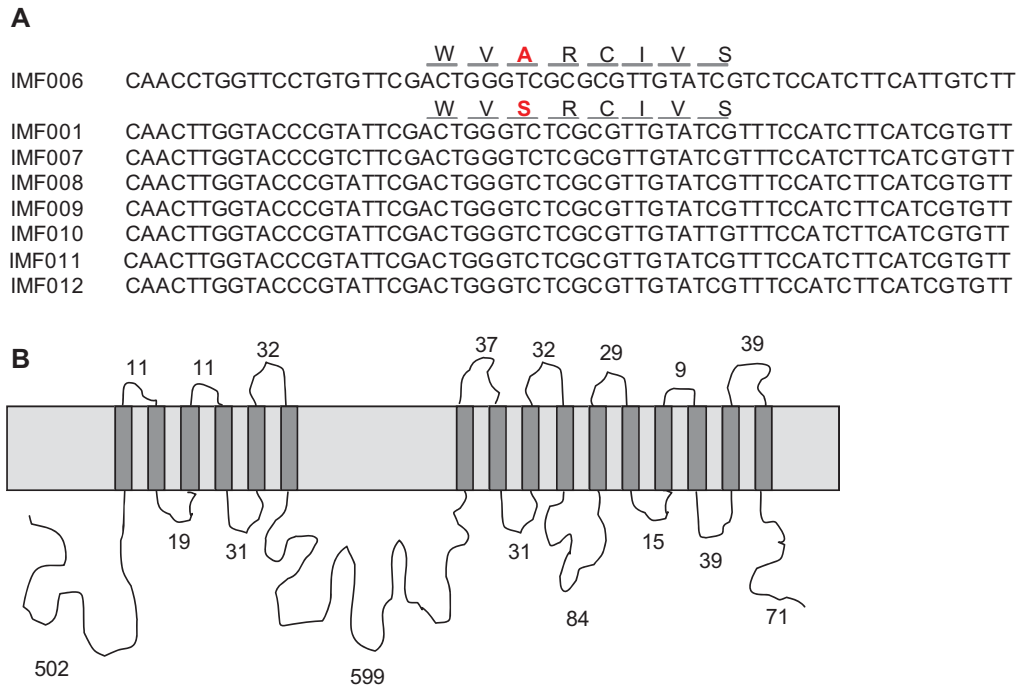

Fig. 4 (A) Schematic topology diagram for the predicted transmembrane AiFKS1 protein. The membrane is represented by a rectangle and the solid black line designates the polypeptide chain with its putative outer and inner loops. The 16 predicted transmembrane helices are indicated by vertical bars. The length number of amino acids of each non-transmembrane domain is shown. This prediction was carried out at the computational server: http://proteinformatics.charite.de/rhythm/. (B) Nucleotide alignment of the designated hot spot 2 region and respective traduction to amino acid sequence of this hot spot.

these fungi and, in one reported clinical case, caspofungin treatment failed to eradicate the infection [5].

In the work presented here we report that several *A. infectoria* clinical strains, among which the one reported by Hipólito and co-workers [5], proved to have *in vitro* susceptibility to caspofungin. A previous study showed

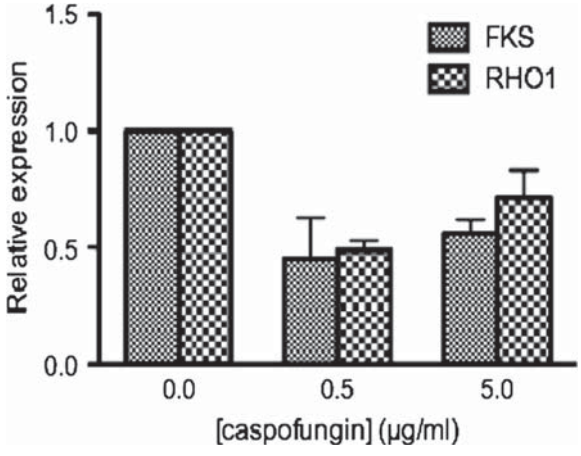

Fig. 5 Quantification of *FKS* and *RHO1* genes expression by real time RT-PCR in *Alternaria infectoria* mycelia incubated in the presence of different concentrations of caspofungin. Data are average ± standard deviation of two independent experiments each performed in triplicate.

that *A. infectoria* is more susceptible *in vitro* to anidulafungin than to caspofungin [36], although the authors do not mention whether the strains were obtained from clinical isolates. One of the major problems found by us was that the *A. infectoria* strains hardly formed conidia, and so we were prompted to use fragmented hyphae in the susceptibility assays. This methodology proved to be very useful in conducting susceptibility assays of filamentous fungi that do not form spores. The results of these assays showed that caspofungin, even at its highest concentration (32 µg/ml) did not prevent fungal growth and, although morphological changes do occur, we never observed the process of bursting of the hyphal tips, reported in *A. fumigatus* [30]. This would appear to suggest that this echinocandin is less efficient in *A. infectoria* than in *A. fumigatus*. One of the observations reported here is that one of the strains harbors a mutation compatible with a lower susceptibility to echinocandins (see below). This strain, according to the information available at the CBS data base (www.cbs.knaw.nl), was isolated from a granulomatous lesion in the arm of an otherwise healthy man. This would seem to indicate that this mutation was not induced in response to caspofungin therapy, as reported for some fungi [20,23]. The MEC value found for all the strains (except IMF006) is compatible with the susceptibility of *A. fumigatus*,

although the clinical correlation studies are limited [21,45]. Arendrup and co-workers [44] described that an *A. fumigatus* isolate that failed to be clinically eradicated by caspofungin, had an *in vitro* susceptible MEC, resistant Etest and a reduced susceptibility in an animal model, stressing the difficulty of correlating *in vitro* results and clinical outcomes [21]. As described above, one of the *A. infectoria* strains included in this study, with a MEC of 1 μg/ml, failed to be clinically eradicated by caspofungin [5].

The glucan-synthase complex has been shown to be composed of the following two proteins; the putative catalytic subunit Fks1p, a large-molecular-size polypeptide with 16 transmembrane domains [8,37], and the regulatory subunit Rho1p, a small-molecular-size GTPase, which stimulates β-1,3-glucan synthase activity in its prenylated form [38]. Here, we report the existence of at least one Fks isoform in *A. infectoria*. The whole genome of *A. infectoria* is not yet been sequenced and as a result we cannot provide definitive confirmation that no other weakly homologous *FKS1* sequences exist in this fungus. We named the gene in this study Ai*FKS1* based on the homology of the FKS family. The AiFks1 sequence is highly homologous to the Fks protein sequences of clinically important fungi such as *A. fumigatus*, *C. albicans* and *C. immitis*, in particular in the predicted cytoplasmic loop region expected to contain the catalytic site. One possible explanation for the increased resistance of *A. infectoria* to caspofungin could be the presence of multiple *FKS* isoforms. All PCR products amplified from *A. infectoria* by using fully degenerate *FKS* primers led to the identification of only one *FKS* sequence. Several filamentous fungi with complete genome sequences, including *N. crassa*, *A. nidulans*, *A. fumigatus*, and *C. neoformans*, have a single *FKS* gene [13]. We also searched for another *FKS* in the genome of *Alternaria brassiucola* (www.jgi.doe.gov/) and of *D. tritici-repentis* (teleomorph *Pyrenophora tritici-repentis*; www.broadinstitute.org) due to the significant homology of the FKS gene but we only found a single FKS in these species.Taken together, these data seem to indicate that Ai*FKS*1 is the only *FKS* gene in *A. infectoria*.

Rho-type GTPases function as key regulators in many cellular processes [11]. The small monomeric Rho-type GTPase, a conserved family within the Ras superfamily, is defined by domains responsible for GTP and GDP binding, plasma membrane localization, and GTPase activity. Rho-type GTPases act as molecular switches: binding GTP activates interaction with downstream effector proteins; hydrolysis of GTP inhibits interaction with effector proteins [39]. In this study a *RHO* gene of *A. infectoria* was also cloned and sequenced. It is known that mammalian RhoA proteins are highly conserved; in fact they hold 90% of identity in their primary sequences [40]. The complete homology between the Rho amino acid sequences from *A. infectoria* and *D. tritici-repentis* (teleomorph: *Pyrenophora tritici-repentis*), both Pleosporaceae, underlines the high conservation throughout the phylogenetic tree for this gene. Of note is also the high homology between these proteins and its homologue from *Homo sapiens* (73%).

It was proposed that mutations conferring echinocandin resistance reside in two hot spot regions of FKS1p (CaFks1 F641- D648 and D1357-L1364), which are highly conserved among FKS genes in different fungal species [18,19,22]. We identified that all the *A. infectoria* strains tested share the same amino acids sequence around the hot spot 1 but in the hot spot 2 the strain IMF006 (CBS 137.9) presents a predicted S1405A substitution which aligns with the amino acid immediately before to the R1357S Fks1p substitution identified in *S. cerevisiae* mutant MS14 and the R1361G substitution of *Candida krusei* strain CLY16038, a clinical *C. albicans* mutant less susceptible to caspofungin in the disseminated candidiasis model [22]. We also detected a lower susceptibility to caspofungin in the strain harbouring this mutation, which exhibits a higher MEC than the other strains without substitution. This result obtained *in vitro* lacks information about the susceptibility of the strain to caspofungin *in vivo*, since the discrepancies between susceptibilities obtained *in vitro* and the concomitant outcome *in vivo* have been widely discussed [3,17].

One of the major conclusions drawn from the present study is that, in *A. infectoria*, low concentrations of caspofungin, lower than the MEC_{90} measured *in vitro*, result in a decrease of *AiFKS* (and *AiRHO* gene expression), as reported by others in an *A. fumigatus*-susceptible strain but not in a resistant strain [44]. The expression of the genes recovered to 80% of the control when the concentration of caspofungin increased above the measured MEC. Recently, the data about genome wide screens in response to echinocandins was reviewed, and in fact, several families of genes are up-regulated in response to sub-MIC/sub-lethal concentrations of caspofungin, as a strategy of adaptative growth [17]. It is a paradox that the exposure to an inhibitor of an essential enzyme leads to a decrease in the transcription of the gene coding for it. This change in the level of transcription of the genes coding for the β-glucan-synthase complex, both for the catalytic and for the regulatory units, strongly indicates a hypothetical influence of caspofungin in the regulation of these genes transcription, independently of its effect due to disturbance of the cell wall. We believe that further studies are required to unravel the physiological significance of this group of data.

Acknowledgements

This study was partly supported by a Merck, Sharp & Dohme, Inc. Medical School Grant (P-1599) and by a funded project by FCT-Fundação para a Ciência e Tecnologia (PTDC/SAU-ESA/108636/2008; co-funded by COMPETE and FEDER). JA was recipient of a pos-doc fellowship within the scope of MSD Medical School Grant (P-1599). CF is a recipient of a Pos-doc Fellowship from FCT-Fundação para a Ciência e Tecnologia (SFRH/BPD/63733/2009). BMAS is a recipient of a research fellowship whithin the scope of the FCT Project PTDC/SAUESA/108636/2008.

The authors acknowledge Dr. Cameron Douglas, of Merck & Co., Inc., for providing protocols, and helpful and stimulating discussions.

Declaration of interest: The authors have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 30 April 2012.

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