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Characterization of *Aspergillus fumigatus* mutants with reduced susceptibility to caspofungin

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Caspofungin acetate (CAS) is a member of a new class of clinically-approved echinocandin drugs to treat invasive aspergillosis. CAS inhibits the activity of β-1,3-D-glucan synthase (GS), thus damaging the fungal cell wall. Although no clinical resistance of *Aspergillus* to CAS has been reported as yet, the development of *in vitro* reduced susceptibility is presumed to be inevitable. By contrast, echinocandin resistance in laboratory strains of *Candida albicans* and *Saccharomyces cerevisiae* has been well documented. To study the potential for clinical resistance in *Aspergillus*, two classes of *Aspergillus fumigatus* mutant strains were isolated that exhibited reduced susceptibility to CAS. In the first class, a site-directed mutation within the target gene (*AfFKS1*, encoding the putative catalytic subunit of GS) was introduced and shown to confer low-level (16-fold) reduced susceptibility. A second class of spontaneous mutants were sensitive to low levels of drug but displayed nearly normal growth above 0.5 μg/ml, suggesting induction of an unknown resistance mechanism. At higher levels of drug ($\geq$16 μg/ml), the mutants displayed partially restored sensitivity. Preliminary studies indicate that neither target site mutations, nor changes in target gene expression are present in these strains, as has been documented for several yeasts. Instead, preliminary results indicate that the molecular mechanism(s) underlying reduced susceptibility of CAS in the *A. fumigatus* strains is novel, possibly due to remodeling of the cell wall components.

Keywords *Aspergillus fumigatus*, caspofungin, drug resistance, *FKS*1, mutant

Introduction

Within the past decade, invasive fungal infections have steadily emerged as a significant cause of morbidity and mortality in immunocompromised patients. An epidemiologic shift in the type of mycosal infection has also been noted, with *Aspergillus* spp. replacing *Candida* as the primary pathogen at some institutions [1]. Established antifungal drugs for life-threatening *Aspergillus* infections target cell wall and membrane proteins. The compounds are aimed at ergosterol, a predominant sterol within fungal cell membranes, and are either fungicidal but toxic to the host (polyenes) or fungistatic and vulnerable to resistance (azoles). The echinocandin caspofungin (MK-991, L-743,872; Merck & Co., Ltd.) is the first of a new class of antifungal compounds with a novel mechanism of action. Caspofungin acetate (CAS) targets the fungal β-1,3-glucan synthase enzyme (GS), thus inhibiting the synthesis of 1,3-β-D-glucan [2,3]. This fungal-specific polysaccharide is a predominant filamentous component of the cell wall of many pathogenic fungi, yet absent in mammalian cells, thus ensuring the compounds inhibit in a potent and specific manner without severe side-effects.

In vitro, CAS has broad-spectrum antifungal activity against *Candida* and *Aspergillus* spp. without cross-resistance to existing agents. The compound exerts prolonged post-antifungal effects and fungicidal activity against *Aspergillus fumigatus* at the sites of hyphal growth [4]. CAS has been shown to have efficacy in animal models of disseminated and pulmonary

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aspergillosis, whilst maintaining an excellent safety profile [5,6]. The safety and tolerability of CAS in the treatment of fungal infections have been evaluated in a number of recent studies, with no serious clinical or laboratory drug-related adverse events reported in the majority of patients [5]. The compound has been approved for use in patients with invasive aspergillosis (IA) who are refractory to or intolerant of other therapies. The absence of antagonism in combination with other antifungal drugs suggests that CAS could be highly effective as part of a combination antifungal therapy [7]. Recent clinical experience with CAS has been summarized in several in-depth reviews [5,8,9].

Owing to their different mode of action and molecular structure, it is unlikely that cross-resistance between azole/polyene antifungals and echinocandins occurs. Clinical resistance to CAS of *Aspergillus* sp. has not been reported, although this could possibly reflect limited drug exposure and small patient numbers. The mutant *A. fumigatus* strains obtained in our laboratory provided an excellent opportunity to investigate the nature of reduced susceptibility prior to the anticipated observation of clinical resistance.

CAS is considered to exert its effect via the FKS1p protein, the putative catalytic subunit of the GS complex [3]. *In vitro* CAS-resistant isolates of *Candida albicans* and *Saccharomyces cerevisiae* have been reported to contain target-site mutations within the *FKS1* gene [2,3]. CAS resistance in *Scharomces cerevisiae* has also been linked to the over-expression of a golgi protein (SBE2p) involved in the transport of cell wall components [10]. In addition, over-expression of the *CDR*2 gene, encoding an ATP-binding cassette transporter, may mediate low level reduced susceptibility to CAS in azole-resistant *C. albicans* isolates [11]. However, recent analysis [12] of a *Candida* isolate collection determined that fluconazole-resistant isolates were susceptible to CAS, indicating that over-expression of drug efflux pumps is unlikely to be a dominant mechanism for conferring resistance to CAS.

The fungal cell wall is a dynamic structure, as constitutive polymers are constantly being chemically modified and rearranged during cell wall biosynthesis. Cellular integrity depends on the proper composition of the cell wall, and is regulated by Pkc1p-mediated signal transduction through a MAP kinase pathway. Although largely unstudied in *A. fumigatus*, the cell wall integrity pathway and control of 1,3-β-D-glucan synthesis have been extremely well characterized in *S. cerevisiae* [13–15]. Despite considerable differences in the protein composition of the cell wall between *A. fumigatus* and *S. cerevisiae* [16], it is presumed such important pathways will be largely conserved. Thus, the yeast studies were used as a starting point to elucidate the novel molecular mechanism(s) underlying reduced susceptibility to CAS in the *A. fumigatus* mutant strains.

FKS-dependent reduced susceptibility to CAS in *A. fumigatus*

Owing to the similar mechanisms observed for yeast and *Aspergillus* in the development of azole and polyene resistance, knowledge of the CAS resistance mechanisms in yeast has been used in the initiation of the *A. fumigatus* study. *In vitro* CAS-resistant isolates of *C. albicans* and *S. cerevisiae* have been reported to contain mutations within specific regions of the *FKS1* gene [2,3]. Investigation of analogous changes in the *AfFKS1* gene (GenBank U79728) has resulted in the generation of an isolate (FKS-S678Y) containing the S678Y change within AfFKS1p. The strain exhibits reduced susceptibility to CAS, with an MEC of approximately 4 μg/ml in liquid RPMI, in comparison to the wild type MEC of approximately 0.25 μg/ml. Growth of isolate FKS-S678Y is also stronger than that of the wild type strain on solid media containing 10 μg/ml CAS (Fig. 1). Growth is comparable to the wild type strain at 25°C, 30°C and 37°C, however at 45°C, strain FKS-S678Y exhibits marginal growth and condiospores are white in appearance. Further characterization of this isolate, and isolates containing other mutations at the *AfFKS1* locus, is underway.

Characterization of isolates with reduced susceptibility to CAS

To investigate additional mechanisms of reduced susceptibility to CAS in *A. fumigatus*, a class of spontaneous mutants was generated by digestion of the cell wall, with subsequent regeneration of spheroplasts on media containing 10 μg/ml CAS. Reduced susceptibility to CAS of the representative mutant strain RG101 was demonstrated by growth on solid media and MIC susceptibility assays (Figs. 1 and 2, respectively). CAS typically inhibits fungal growth at the hyphal tips; thus, CAS-sensitive strains of *A. fumigatus* characteristically form rosette-like structures when grown in the presence of the drug (Fig. 2B) [4]. By comparison, the mutant strain exhibits uninhibited growth at high concentrations of the compound (Fig. 2A). The mutant isolate displays a biphasic growth phenotype, with CAS sensitivity observed at low levels of drug, but nearly uninhibited growth above 0.5 μg/ml, suggesting induction of some mechanism(s). At higher levels of drug

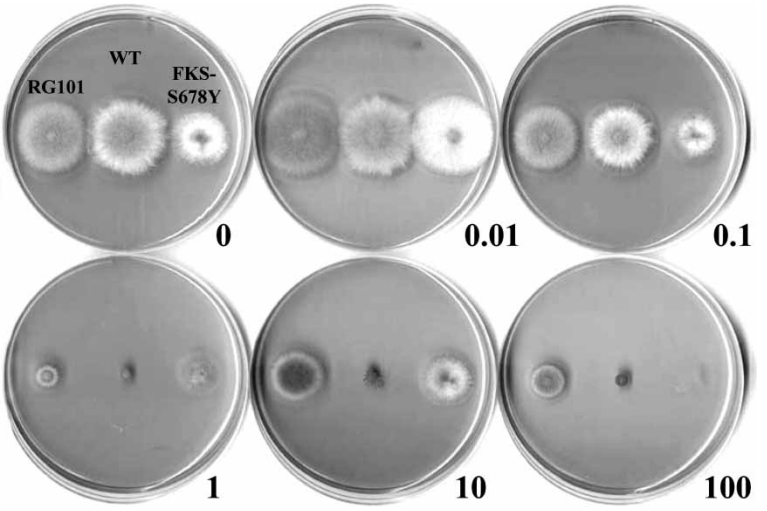

Fig. 1 *Aspergillus fumigatus* wild type and reduced-susceptible mutant strains, on AM3 media containing varying CAS concentrations (μg/ml). All plates contain strains (from left): RG101, wild type strain (WT) and FKS-S678Y. Strains RG101 and FKS-S678Y display reduced susceptibility to CAS. Growth shown is after two days at 37°C.

(>16 μg/ml), the mutant displays restored sensitivity (Figs. 1 and 2).

The prolific growth of the mutant strains in the presence of CAS, in comparison to the hindered growth of the susceptible strain, was confirmed by growth studies on solid media (Fig. 1). It can also be observed that strain RG101 displays an increased level of reduced CAS-susceptibility, in comparison to strain FKS-S678Y (Fig. 1). No cross-resistance of strain RG101 to other antifungals, including amphotericin, itraconazole and nikkomycin, has been observed. It is intriguing to note that the mechanism(s) of reduced susceptibility to CAS in the mutant isolates appears to be CAS-specific, as susceptibility to other echinocandins is retained [D. Denning, personal communication]. The mutant strains exhibited virulence in an animal model of disseminated aspergillosis, comparable to the wild type strain [C.M. Douglas, personal communication]. The reduced susceptibility phenotype appears to be stable, as determined by passaging of the mutant isolates on drug-free media (approximately 30 passages carried out to date). Growth of the reduced susceptible strains on drug-free media at different temperatures is comparable to the wild type, indicating the mutant strains do not appear to be temperature sensitive. All isolates were confirmed to be *A. fumigatus* by molecular beacon analysis with a probe specific to the *ITS2* region.

AfFKS1 independent mechanism

To ascertain the mechanism of reduced susceptibility to CAS in the mutant strains, sequencing of the target gene (*AfFKS1*) was undertaken. CAS resistance in *C. albicans* and *S. cerevisiae* has been mapped to mutations within the target gene [24]. However, no sequence mutations within the (6.5 Kb) *AfFKS1* gene were observed in the *A. fumigatus* mutant strains. Subsequent analysis with molecular beacon technology revealed no significant CAS-induced differences in *AfFKS1* gene expression between the mutant and wild type strains. Thus, it would appear that the mechanism(s) employed by these *A. fumigatus* mutant strains is independent of the *AfFKS1* gene. Congruent with these results, the GS enzyme activity from the mutant isolates has been shown to be present in levels comparable to the wild type isolates, and the enzyme itself is sensitive to CAS [J. Nielsen, personal communication].

Preliminary analysis of cell wall components

It was postulated that the method used to generate the mutant strains may have altered the composition of the cell wall through induction of the cell wall integrity pathway, ultimately conferring reduced susceptibility to CAS. Compensatory over-expression of cell wall components to maintain cellular integrity has been reported in fungi and yeast [18,25], thus providing some candidate genes for initial investigation in the *A. fumigatus* mutant strain RG101. For example, *FKS1* deletion strains of *S. cerevisiae* have been reported to over-express the *FKS2* and chitin synthase genes [18]. *S. cerevisiae* and *C. albicans* both contain isoforms of the *FKS* gene, which are differentially expressed under certain conditions. However, only one *FKS* gene has been reported in *Aspergillus* species to date [26], the essential nature of which was demonstrated recently [27]. An *A. fumigatus* 'glucan synthase-like' (GSL) sequence (GenBank AF007067) which displays 56%

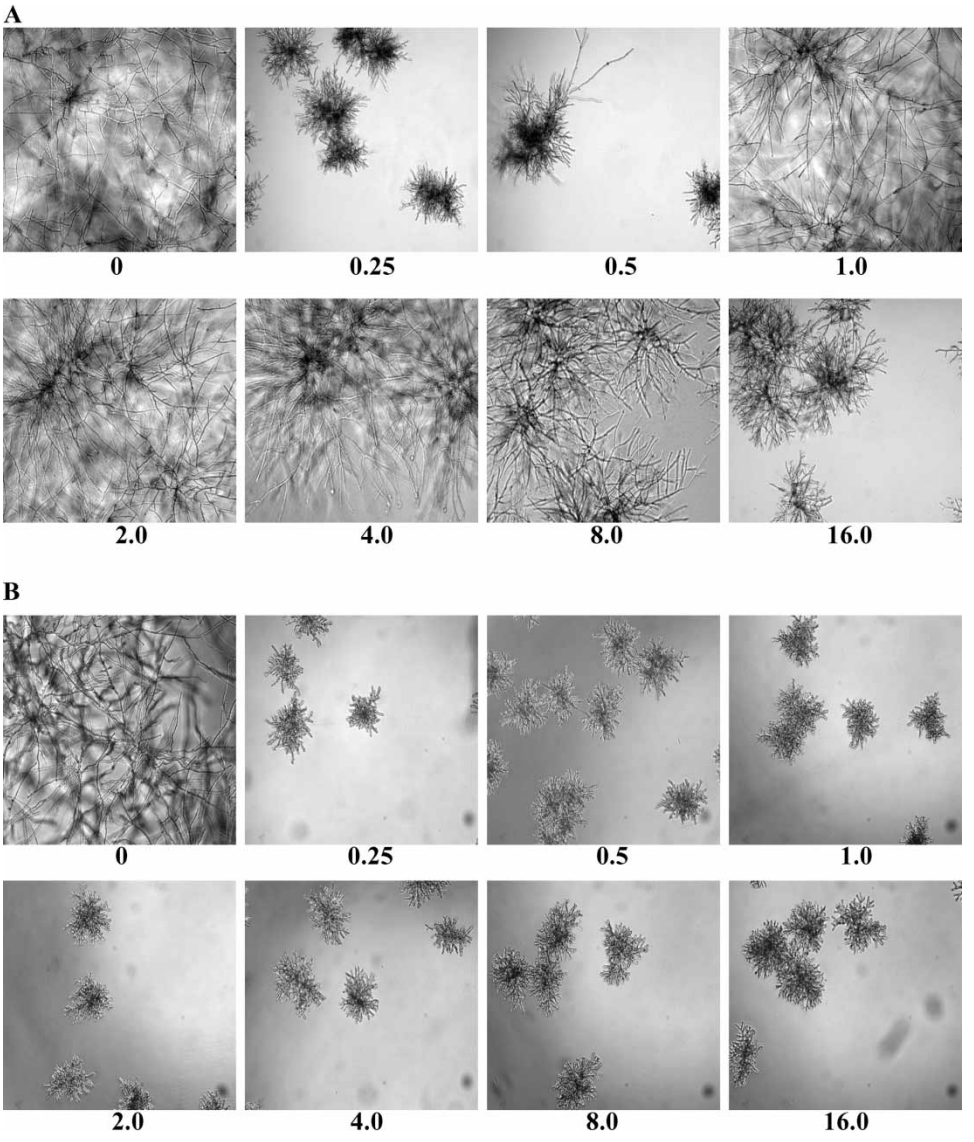

Fig. 2 Microdilution assay for reduced-susceptible mutant (A) and wild type (B) *Aspergillus fumigatus* isolates. Growth is shown after 24 hours growth in RPMI media. CAS concentrations are given in μg/ml.

homology to the *AfFKS1* gene was included in the analysis.

However, preliminary real time RT-PCR results have indicated that gene expression of other cell wall components, such as α-1,3-glucan and chitin synthase, is not induced in the mutant strain in response to CAS. Expression profiles of the *A. fumigatus GSL* gene and other cell wall (*RHO1*, *FOS1*) or transport (*SBE2*, *KEX2*) related genes were also not significantly altered in the mutant strains. In addition, sequence analysis was carried out for several of these genes. Although these studies have not yet been concluded, no significant differences in gene expression nor amino acid sequence have been observed which could account for the reduced CAS-susceptibility phenotype of the *A. fumigatus* mutant strains. No significant differences in the chitin and β-1,3-glucan distributions for the mutant and wild type strains was observed upon Calcofluor staining. Although preliminary cell wall carbohydrate analysis suggested that glucan and chitin levels may be slightly elevated in the mutant strains, results were inconclusive [J. Nielsen, personal commumication]. A more extensive cell wall analysis of the mutant strains is currently underway.

Differentially expressed genes identified by SSH

A more global approach was undertaken to identify the mechanism(s) employed by the mutant strain. The

biphasic growth phenotype exhibited by strain RG101 (Fig. 2A) indicated that the reduced susceptibility of the strain may be due to changes in gene expression that were drug inducible. Subtractive suppressive hybridization (SSH) was carried out to isolate genes that were differentially expressed by the mutant strain in response to CAS. Approximately 100 differentially expressed cDNA transcripts were cloned and analysed by DNA sequencing; 90% of the predicted ORFs shared identity with proteins from other fungi, including *Aspergillus* sp. and *S. cerevisiae*. The predicted functional classes of the cDNA transcripts generated by SSH include the following: metabolism and bioenergetics, cell wall maintenance, signal transduction, RNA and protein processing, and transport (Fig. 3). As postulated, induction of a cell wall integrity pathway and expression of downstream cell wall components is likely in the mutant strain in response to CAS, as evidenced by signal transduction histidine kinases, SWI/SNF complex components, glucoamylase I and GPI-like proteins. Increased RNA and protein processing, demonstrated by expression of RNA helicase, tRNA synthase and proteosome subunits, may also reflect remodeling of the cell wall composition upon exposure to CAS.

Miniarray analysis

To investigate more broadly the expression of relevant genes that may contribute to the reduced CAS susceptibility of the mutant strains, miniarray analysis of 220 target and control genes was performed. In addition to the cDNA transcripts identified by SSH (denoted 'MCI' clones), PCR amplicons were prepared for genes putatively involved in the cell wall integrity pathway, cell wall maintenance [D. Denning, personal communication] and stress response. Additional genes reported to be differentially regulated in *S. cerevisiae* in response to CAS [18–20] and spheroplasting [21], or known to interact with *FKS1* [22] were also included. Preliminary results indicated increased expresssion of 28 genes for the mutant strain RG101 in the presence of CAS, in comparison to the wild type strain (Table 1). These genes encode structural components of the cell wall (*AGS2*, *CWP1*, *MCI 36*), cell wall biosynthesis enzymes (*KRE1*, *MCI 35*), and signal transducers (*MAPK*, *MCI 106*). Expression of genes encoding transport-related proteins, such as MDR1p and MDR4p, was also induced in the RG101 strain. Furthermore, CAS-enhanced transcription of genes involved in RNA processing, general metabolism and growth was observed in the mutant strain. No significant down-regulation of genes was observed for the mutant strain, relative to the wild type strain. In addition, no significant changes in gene expression were observed between the mutant and wild type strains, in the absence of CAS.

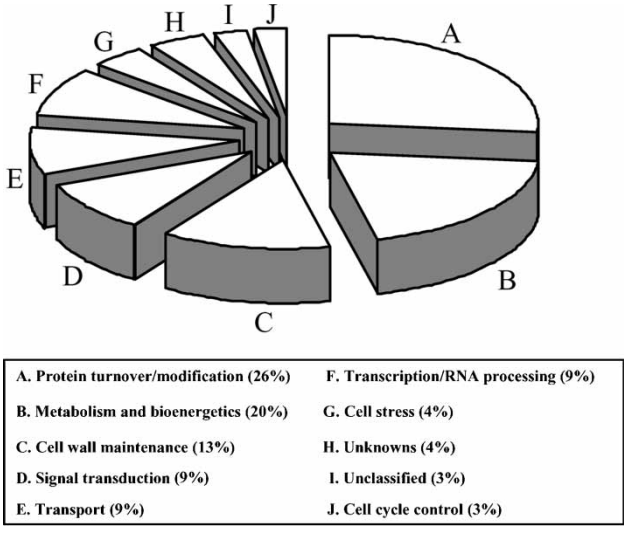

Fig. 3 Functional classification of cDNA transcripts isolated by SSH. The transcripts are differentially expressed in response to CAS, for strain RG101. The percentages indicate distribution of predicted functions in broad functional categories.

These results validated the importance of genes involved in cell wall biosynthesis/modeling and transport in the reduced susceptibility response, and support the observation that cell wall components may be rearranged in the mutant strains. Homologues of *S. cerevisiae* genes involved in the cell wall integrity pathway [13,20] were not observed to be over-expressed; however, it is interesting to note that a GTP exchange factor with similarity to *S. cerevisiae ROM2* (an upstream regulator of *RHO1* in the cell wall integrity pathway) is induced 5.2-fold (*MCI 39*, Table 1). Taken together, these results suggest that exposure of the mutant strain to CAS might trigger some type of signaling pathway, resulting in the up-regulation of cell wall proteins.

Alternatively, the over-expression of genes encoding transport proteins (e.g. *MDR1* and *MDR4*) may reflect either specific drug and/or metabolite transport; a postulation which may be supported by the observation that the mechanism(s) of reduced susceptibility to CAS in the mutant isolates appears to be CAS-specific. It is worth noting that expression of the genes *SBE2* and *CDR2*, encoding golgi and ABC-type transport proteins (respectively), was not induced in the mutant strains. Over-expression of these genes has been reported to confer CAS resistance in yeast [10,11]. Not all of the MCI clones identified by SSH were

Table 1 *Aspergillus fumigatus* genes up-regulated by CAS in the reduced-susceptible mutant RG101, relative to the wild type strain

Gene	Putative Function of Predicted ORF	Fold induction[a]
Cell-wall related and transport		
MCI[b] 39	Putative GTP exchange factor/ major facilitator superfamily protein	5.21
MAPK	Has homology to *A. nidulans* MpkCp and *S. cerevisiae* MAPKp. Putatively involved in the cell wall integrity pathway.	3.65
MDR1	Multi drug resistance protein 1.	3.50
KRE1	Involved in cell wall beta-glucan assembly.	3.08
OSM1	MAPK involved in osmoregulation, induced under stress conditions. Homologue of *S. cerevisiae* HogAp.	2.98
MCI 35	Putative glucoamylase I.	2.93
MCI 20	cAMP-dependent protein kinase catalytic subunit/zinc finger protein/copper transporter.	2.83
MCI 55	Metaxin 1-binding protein/glutathione S-transferase-like protein/glycosyltransferases.	2.50
MCI 106	BolA-like protein involved in signal transduction, possibly induced by stress.	2.45
MDR4	Multi drug resistance protein 4.	2.22
CWP1	Cell wall protein 1. GPI-associated, involved in glycosylation.	2.09
MCI 36	Homology to Crh-like protein and Avicelase III (*A. nidulans*), also *S. cerevisiae* cell wall proteins, including WSC2p and 4p, SCW11p.	2.08
MCI 9	Probable zinc transporter/ABC transporter.	2.01
AGS2	α-glucan synthase 2, structural component of the cell wall.	2.00
General metabolism and growth		
MCI 63	Methionyl-tRNA synthetase.	3.46
MCI 49	Virulence-related surface glycoproteins/guanine nucleotide binding protein.	3.32
MCI 27	Glutamine amidotransferase.	3.29
MCI 61	Gamma-glutamylcysteine synthetase.	3.17
MCI 80	ADP/ATP translocase or Carbon catabolite repressor protein.	3.04
MCI 51	Disulfide isomerase.	2.89
MCI 50	Beta-tubulin folding cofactor C.	2.44
MCI 38	Putative transmembrane proton channel.	2.00
RNA processing		
MCI 100	Set domain protein, involved in chromatin-mediated gene regulation.	4.93
MCI 70	Probable ribosomal protein L12.	3.49
MCI 41	*Aspergillus* mitochondrial ribosomal RNA.	2.04
MCI 37	Putative condensin complex component.	2.16
Unknown		
ECM21	Non-essential protein of unknown function.	2.94
ECM4	Non-essential protein of unknown function.	2.73

[a]That is, the average of two arrays (dye flip replicate).
[b]MCI (Mutant Caspofungin Induced) denotes cDNA clones identified by SSH.

observed to be differentially expressed in the mutant strain in response to CAS, probably because the normalization criteria eliminated some genes, eg. expression had to change at least two-fold between the two conditions.

A more global approach to analysing gene expression is currently underway with microarrays containing a full representation of the *A. fumigatus* genome, along with a more extensive analysis of the cell wall, to fully understand the basis of the reduced CAS susceptibility of the mutant strains.

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Appendix 1

Control genes for *A. fumigatus* miniarray

Gene	Gene function
ACC1	Acetyl-CoA carboxylase [28]
δ actin	Component of cytoskeleton [28]
ADP1	Shows homology to ATP-dependent permease [29]
α tubulin	One of the main components of hyphal tubulin [30]
β tubulin	One of the main components of hyphal tubulin [30]
δ tubulin	Homologue of microtubule interacting protein of *A. nidulans*, essential for mitotic spindle formation [30]
GLN4	Glutaminyl-tRNA synthetase [29]
GSDA	Glucose-6-phosphate dehydrogenase, homologue of *S. cerevisiae* ZWF1 [29]
RPL3	Ribosomal protein L3 [28]
RPS16	Ribosomal protein [28]
VPS13	Vacuolar protein sorting-associated protein [29]