Chitin synthases with a myosin motor-like domain controls the resistance of

*Aspergillus fumigatus* to echinocandins

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Abstract

Aspergillus fumigatus has two chitin synthases (AfCSMA and AfCSMB) with a myosin motor-like domain (MMD) arranged in a head-to-head configuration. To understand the function of these chitin synthases, single and double csm mutants were constructed and analysed. Although there was a slight reduction in mycelial growth of the mutants, the total chitin synthase activity and the cell wall chitin content were similar in the mycelium of all the mutants and the parental strain. In the conidia, chitin content in the ΔcsmA cell wall was less than half the amount found in the parental strain. In contrast, ΔcsmB and unexpectedly the ΔcsmA/ΔcsmB mutants did not show any modification of the chitin content in their conidial cell-walls. In contrast to the hydrophobic conidia of the parental strain, conidia of all the csm mutants were hydrophilic due to the presence of an amorphous material covering the hydrophobic surface-rodlet layer. The deletion of CSM genes resulted also in an increased susceptibility of resting and germinating conidia to echinocandins. These results show that the deletion of the CSMA and CSMB genes induced a significant disorganisation of the cell wall structure even though they contribute only weakly to the overall cell wall chitin synthesis.
Introduction

Chitin, a microfibrillar homopolymer of β-(1,4)-linked N-acetyl-glucosamine (GlcNAc) residues, is one of the major components of the fungal cell wall, contributing to the shape and mechanical strength of the fungal cell. Since this polymer is essential for fungal growth and development, its synthesis has been studied for decades. Moreover, it has been considered as an excellent target for the design of new antifungal agents (5). Chitin synthesis occurs at the plasma membrane with the extrusion of nascent chitin-chains into the cell wall space. Chitin synthases (CHS), the proteins involved in this process are highly variable with a conserved catalytic domain bordered by transmembrane regions. Based on the sequence of their conserved regions, CHS-proteins have been classified in two families that include seven classes (Supplemental Figure 1) (38, 41). Family I include classes I to III while classes IV to VII belong to the Family II (9, 55). Both families are structurally different: in Family I, the catalytic site is bordered by transmembrane regions on each side, whereas in Family II, the central protein core is bound to the membrane through multiple helices at the C-terminus (32, 41).

In fungi, the number of CHS genes varies from one (class III in Encephalitozoon cuniculi) to three (Saccharomyces cerevisiae, classes I, II, IV) or >10 in filamentous fungi encompassing all the CHS classes; the number of CHS genes and families found in fungi being mostly correlated with the amount of cell wall chitin (25, 31, 50). In addition, classes III, V, VI and VII have only been identified in filamentous fungi, suggesting that these enzymes may play a role in hyphal growth. The function of the different CHS is only understood in S. cerevisiae where it has been shown that each class of CHS-proteins has different function. Chs1p acts as a repair enzyme when mother and daughter cells separate (6). Chs2p synthesizes the chitin of the septum between mother and daughter cells (44). Chs3p is responsible for the synthesis of
the chitin present in the lateral cell wall and at the chitin ring at the base of an emergent bud (43). In other fungi, the function of all individual CHS-proteins and their specific involvement and interactions in the synthesis of the cell wall chitin remains poorly understood. It is especially puzzling to see that some chitin synthase mutants such as the chs1 of *Neurospora crassa* or the chs3 of *Exophiala dermatitidis* show a reduction in chitin synthase activity *in vitro* that can reach 90% of the total activity without any reduction in cell wall chitin content; in contrast, chs4 mutants of *N. crassa* and *E. dermatitidis* with an amount of chitin between 50 and 75% of the wild-type strain had a normal chitin synthesis activity *in vitro* (15, 54).

Eight chitin synthase genes were identified in *Aspergillus fumigatus* (*CHSA, CHSB, CHSC, CHSG, CHSF, CSMA* (earlier called *CHSE*), *CSMB* and *CHSD*). To date, six simple and three double mutants were constructed (G/C, A/C, G/E) (32-35, 40). Inactivation of all the Family I genes *CHSA* (class I), *CHSB* (class II), *CHSC* and *CHSG* (class III), individually, did not lead to any growth phenotype with the exception of *CHSG*. Characterisation of Family II *CHS* remained incomplete in *A. fumigatus*; only *CSMA* (class V) and *CHSD* (class VI) were analysed (32). Disruption of *CSMA* (class V) leads to an altered growth phenotype (poor conidiation, reduction in the colony radial growth rate and decrease in the chitin synthase activity) whereas the deletion of *CHSD* (class VI) did not result in any phenotype modification (2, 32). Although class V and class VII enzymes are required for correct morphogenesis in several filamentous fungi, there was no direct experimental evidence of the role of these gene products in the polysaccharide synthase activities as well as in the modification of the structural organisation of the cell wall chitin (20, 21, 24, 27, 29, 55, 56). It has been only shown to date that these enzymes have a conserved myosin-17 MMD that drives them to the tip of the hyphal cell (19, 42).
To understand better the function of *A. fumigatus* MMD-chitin synthases, single and double csm mutants were constructed and analysed. Although CSMA and CSMB are arranged in a head-to-head configuration, their functions in cell wall construction were found to be different. Class V CsmA CHS-protein was involved in the conidial chitin synthesis. In contrast, class VII CsmB CHS-protein did not play a significant role in the chitin synthesis in the conidial cell wall. Even though the deletion of CSMA and CSMB was not associated to a modification of the mycelial chitin level, the overall organisation of the cell wall polysaccharides was perturbed leading to an increased sensitivity to antifungal drugs.

**Material and Methods**

**Strains and culture conditions**

*Aspergillus fumigatus* strains used in this work are listed in the Supplemental Table 1. They were grown at 37°C in either *Aspergillus* minimal medium (AMM) (11) containing 1% glucose and 5 mM ammonium tartrate, YG (1% glucose, 0.5% yeast extract, 1% trace elements), Sabouraud (2% glucose, 1% neopeptone) (Difco), PDA (potato dextrose agar) (Difco) or 2% Malt agar (Cristomalt). When necessary, 6% KCl or 1 M sucrose was added to the media to enhance conidiation. Conidia were collected from agar media plates after 10-days of growth at 37°C, using water containing 0.05% Tween 80.

**Deletion of CSM genes**

Two different strategies were used to create single and double deletion of the CSMA and CSMB genes. Strategy 1: plasmids pCJ-E4, pCJ-Eb4 and pCJ-D2 were used for the construction of the ΔcsmA, ΔcsmB and ΔcsmA/ΔcsmB mutants, respectively. These plasmids carry a DNA fragment designed to delete most of the ORFs of the CSMA and CSMB genes. The strategy outlined in the Supplemental Figure 2 was used to produce strains with non-
functional, disrupted *AfCSM* genes. The knockout Δcsm plasmids (pCJ-E4, pCJ-Eb4 and pCJ-D2) were constructed in the pGEM®T vector (Promega), which contained two fragments of *AfCSM* genes separated by the 3.88 kb *hisG-pyrG-hisG* cassette obtained from the pPYRG2 plasmid (51). The two *AfCSM* fragments were obtained by PCR with *A. fumigatus* genomic DNA as a template and two sets of primers (AfV-A1/AfV-A2 and AfV-A5/AfV-A6) flanked by restriction enzymes for cloning (see Supplemental Table 1). For the disruption of CSMA, protoplasts of a CEA17 strain of *A. fumigatus* were transformed using a 7.4 kb linear fragment released from pCJ-E4 by *Not*I digestion. The construction of the ΔcsmB strain was carried out by transforming CEA17 protoplasts with a 7.1 kb linear DNA fragment (obtained by PCR using the primers AfVB-B1/AfVB-B2 and AfVB-B5/AfVB-B6) released from pCJ-Eb4 by *Not*I digestion. For the construction of the double mutant ΔcsmA/ΔcsmB a simultaneous deletion of both genes was carried out using a 7.2 kb linear DNA fragment (obtained using the primers AfVB-B1/AfVB-B2 and AfV-A5/AfV-A6) released from pCJ-D2 by *Not*I digestion. The transformation of *A. fumigatus* CEA17 strain using pCJ-E4, pCJ-Eb4 or pCJ-D2 was accomplished using the protoplast procedure described previously (51). After 3-days at 37°C, the pyrG+ transformants obtained were isolated from the AMM plates and were analysed by PCR with different sets of primers, with one oligonucleotide primer inside the *hisG-pyrG-hisG* deletion cassette and another one upstream from the ORF of the gene of interest using genomic DNA obtained from spores as described (51). Integration of the cassette at the correct locus was confirmed by Southern blot with genomic DNA digested by *Xmn*I for ΔcsmA, *Nco*I for ΔcsmB and *Bgl*II for the double mutant (Supplemental Figure 2).

Strategy 2: Due to unexpected results with the double ΔcsmA/ΔcsmB mutant it was decided to undertake another replacement strategy in a different genetic background. The ΔcsmA/ΔcsmB double deletion mutant was constructed in CEA17_ΔakuBKU80 background (13) using the β-
rec/six site-specific recombination system (18). The self-excising β-rec/six blaster cassette containing the hygromycin resistance marker was released from the plasmid pSK529 via FspI restriction enzyme. Using the GeneArt® Seamless Cloning and Assembly (Life technologies, Carlsbad, CA 92008 USA) the csmA and csmB replacement cassette containing the marker module flanked by 5’ and 3’ homologous regions was generated and cloned in the pUC19 vector. The corresponding replacement cassette of 6796pb for csmA and 6806pb for csmB were released from the resulting vector via EcoRV and FspI respectively. The CEA17ΔakuB*KU80 parental strain was transformed with the csmA replacement cassette by electroporation. Transformants obtained were analysed by diagnostic PCR with oligonucleotides forw csmA and Sv630 (Supplemental Table 1). The ΔcsmA deletion mutant obtained was cultivated in presence of 2% xylose-containing minimal medium that allows the excision of the selection marker, by recombination of the six recognition regions. The proper integration of the csmA replacement cassette and the excision of the selection marker in the ΔcsmAx strain were then verified by Southern blot analysis (Supplemental Figure 3). To obtain the ΔcsmA/ΔcsmB double deletion mutant, the csmB replacement cassette was transformed in the ΔcsmAx recipient strain. Transformants obtained were analysed by diagnostic PCR with oligonucleotides Forw csmB and Sv630 and Southern blot analysis (Supplemental Table 1).

Construction of revertant strains

Complementation of the Δcsm mutants was obtained by a transformation strategy using a wild-type (WT) copy of the genes in the recombinant plasmid pPYRGQ3 which contain the pyrG resistant marker. Spontaneous pyrG− fungal strains from a pyrG− independent clone were selected on AMM containing uracil (0.05%), uridine (0.12%), and 5-fluoroorotic acid (1 mg/ml). These pyrG− fungal strains were then used to generate the complemented strains.
using primers shown in the Supplemental Table 1. ORFs of the genes CSMA and CSMB were amplified from genomic DNA of the CEA17 \textit{pyrG}^+ (AF14) strain and inserted into pPYRGQ3 that had been digested with \textit{XbaI} and \textit{XmaI} to yield pPYRQ3\_CSMA and pPYRQ3\_CSMB. These plasmids were linearized with \textit{NotI} and transformed into the correspondent \textit{pyrG}^- strains of \textit{A. fumigatus}. The presence of the WT copy of the genes was confirmed by PCR and Southern Blot using the probes amplified with the primers sE\_F/sE\_R for \textit{CSMA} complementation and sEb\_F/sEb\_R for \textit{CSMB} complementation (Supplementary Figure 4).

DNA isolation and hybridization

DNA was isolated from \textit{A. fumigatus} using the extraction procedure described by Calera and co-workers (7). For Southern blot analyses, 10 µg of DNA per lane was loaded onto a 0.8% agarose gel and transferred by capillarity to positively charged nylon membranes following standard protocols. Probes for \textit{AfCSMA} and \textit{AfCSMB} were obtained by restriction enzyme digestion of the appropriate clones. The Rediprime™ Random Prime Labelling System Kit (Amersham) was used to label DNA probes according to the manufacturer’s instructions.

Real-time RT-PCR

Vegetative mycelium was obtained after 16 h of culture in a Glucose (3%)-YE (1%) liquid medium. Mycelial growth (for 24 h) on malt agar covered with a cellophane membrane (DryEase cellophane, Invitrogen) was corresponding to the onset of conidiating morphotype. Fungal material were disrupted with 0.5 mm diameter glass beads in 500 µl and then RNA was isolated as described earlier (36) or by using the QIAGEN RNeasy® Mini Kit. Quantitative PCR assays were performed as described in Mouyna et al. (36). The expression ratios were normalized to \textit{TEF1} expression, and calculated according to the $2^{-\Delta\Delta C_{T}}$ method (28).
To verify the absence of genomic DNA contamination, negative controls in which reverse transcriptase was omitted were used for each gene set. Three independent biological replicates were performed. The expression of the eight chitin synthase genes (CHSA-G) has been analysed. Primers used for these genes and the gene accession numbers are given in Supplemental Table 2.

Antifungal assays

Ten-fold dilutions, starting at 2x10^6 spores as the highest concentration, were spotted onto YG plates containing different concentrations of echinocandins (caspofungin, anidulafungin and micafungin), calcofluor white, itraconazole, voriconazole or amphotericin B. Plates were incubated for 48 h at 37°C in a humid atmosphere. Antifungal activity was also assessed in YG/RPMI-MOPS liquid medium using the resazurin method (10). The effect of nikkomycin Z was tested both by CLSI M38-A2 protocol and rezasurin method (10).

β-(1,3)-glucan synthase (GS) and chitin synthase (CS) activity assays

GS activity was measured according to a previously described procedure using an excess of GTPγS (4). CS activity measurements were carried out with crude membrane extracts obtained as follows: flasks with 200 ml of Sabouraud medium were inoculated with 5x10^5 conidia per ml of the different strains and incubated 17 h at 37°C in an orbital incubator. Mycelia were collected by filtration under vacuum, washed with water and disrupted in a MSK (Braun) cell homogenizer in 50 mM Tris–HCl buffer (pH 7.5) containing 50 mM EDTA and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 1 min at 4°C in the presence of glass beads (0.45 mm diameter). The disrupted mycelial suspension was centrifuged (8000g, 10 min) and the supernatant was centrifuged for 35 min at 100000g. Membranes were then washed in 50 mM Tris–HCl (pH 7.5) containing 5 mM magnesium acetate, centrifuged again.
for 35 min at 100,000g and re-suspended in the same buffer containing 30% glycerol and stored at -80°C. CS activity was measured by the incorporation of UDP-N-acetyl-glucosamine (UDP-GlcNAc) into chitin with trypsin treatment, using a modification of the protocol described by Choi and Cabib (8). The assay system for CS activity consisted of 50 μg of membranes, 32 mM GlcNAc, 1.1 mM UDP-[14C]-GlcNAc [293 mCi mmol⁻¹; Amersham Life Science] 4.3 mM magnesium acetate in 30 mM MES (pH 6.5) in a total volume of 50 μl. For the proteolytic activation step, 2 μl of trypsin (1-3 mg/ml) was added to the reaction medium and proteolysis activation was stopped after 15 min of incubation by the addition of soybean trypsin inhibitor solution. Mixtures were incubated at 30°C for 90 min. Newly synthesized chitin was determined by measuring the radioactivity incorporated into the insoluble material after the addition of 10% trichloroacetic acid and filtration through GF/C glass-fiber filters (Whatman). The radioactivity was counted in a Wallac 1409 scintillation apparatus. Specific activity was expressed as nmol of GlcNAc incorporated per hour per milligram of protein.

Carbohydrate analysis of the cell wall fractions

Parental (WT) and chitin synthase mutant conidia were either used directly or grown in medium containing 3% glucose and 1% yeast-extract (YE) for 24 h/36 h for the cell wall polysaccharide analyses. The monosaccharide composition in the cell wall fractions was determined as described earlier (39).

Atomic force microscopy

AFM images were obtained in contact mode at room temperature (20°C) in PBS, using a Nanoscope V Multimode atomic force microscope with oxide-sharpened microfabricated Si3N4 cantilevers of 0.01 N/m spring constants (Bruker, Santa Barbara, CA). For live cell imaging, conidia were immobilized by mechanical trapping into polycarbonate porous
membranes of 3 µm diameter (it4ip). After filtering a cell suspension (200 µl; 2.5x10^6 cells/ml), the filter was carefully rinsed in PBS, cut (1x1 cm) and attached to a steel sample puck using a small piece of adhesive tape, and then the mounted sample was transferred into the AFM liquid cell. The imaging force was kept as low as possible (250 pN) to minimize sample damage (14).

Light microscopic analysis of the fungal morphotypes
Fungi grown for 3 h up to several days on a 2% malt agar were observed by light microscopy. Measurement of the size of conidia and intercalary hyphal cells as well as the width of the mycelium (number of hyphae measured for the width (n) = 30) was performed after staining of the mycelium with calcofluor white (aqueous solution at a final concentration, 5 µg/ml) concentration. Resting and germinating conidia were stained with Trypan blue and calcofluor white (0.4% and 5 µg/ml, respectively, for 5 min). The percent of stained fungal cells is an index of cell wall permeability since none of these dyes was able to penetrate a wild-type conidium.

Extraction of conidial surface hydrophobin (RodAp)
Freeze dried conidia were incubated with 48% hydrofluoric (HF) acid (72 h, 4°C). The extract obtained was dried under N₂, reconstituted in H₂O (1) and the protein was quantified using Bio-Rad protein assay reagent according to the manufacturer’s instructions. An aliquot was subjected to SDS–PAGE (15%) and the proteins were visualized by silver staining.

Conidiation and germination
Conidial suspensions (100 µl, 10⁶/ml) were inoculated into three tubes of Malt agar (2%) or Malt agar containing 6% KCl (10 ml/tube). After 10-days at 25°C, conidia were recovered.
with 2 ml water containing 0.01% Tween 20 and counted using haemocytometer. The percentage germination was quantified after spotting 5x10³ conidia on Sabouraud agar medium or PDA spread over the glass slides, incubating at 37°C and counting the germ tubes microscopically for every one hour between 7-12 h.

Statistical analysis
At least three biological replicates were performed per experiment; the statistical significance of the results was evaluated by a one-way variance analysis using the JMP1 software (SAS Institute, Cary, NC, USA).

Results
Sequence analysis of the two CHS genes with a myosin motor-like domain (MMD) in Aspergillus fumigatus and construction of single and double mutants
BLAST analysis with the class V gene of A. nidulans showed that two orthologous CHS genes with a myosin motor-like domain were located nearby in the chromosome 2 of A. fumigatus. For a better homology with the A. nidulans nomenclature, the genes AFUA_2G13440 (=CHSE) and AFUA_2G13430 were named respectively AfCSMA and AfCSMB (viz., chitin synthase with a myosin motor-like domain A and B, respectively). AfCSMA and AfCSMB were arranged in a head-to-head configuration within the A. fumigatus genome at a distance of 3221 bp between their translational start points (Figure 1). The analysis of the DNA sequence of the CSMB gene revealed an ORF of 5446 bp encoding a 1756 amino-acid polypeptide organized in four exons interrupted by three introns. Originally described as CHSE (2), the full-length of the CSMA gene was 5740 bp and encoded a protein with 1848 amino acids. The proteins encoded by CSMA and CSMB genes showed only an overall similarity of 25% but presented Family II chitin synthase domains (CSD) at their C-
teminal ends that displayed 60% similarity between them. In their N-terminal ends, the CsmA and CsmB proteins had a myosin motor-like domain (MMD), which displayed only 11% similarity. The ATP-binding motifs, such as the P-loop [GXXGXGK(T/S)], Switch I [TASKAG] and Switch II [DFPGF] in the CsmAp-MMD, thought to be essential for ATPase and motor activities (45, 48), were not conserved in the MMD of CsmBp. This head-to-head configuration as well as the absence of ATP binding motifs has previously been described for CSM orthologs in other ascomycetes (30, 31, 45).

Deletion of the CSMA and CSMB genes was performed using two different strategies (Supplemental Figures 2 and 3). The integration of the disrupting cassette at the right locus for the mutant as well as the ectopic integration of a copy of the CSMA and CSMB genes in the complemented strains was verified by Southern blots. All the mutants and the revertants constructed are shown in the Table 1. In order to confirm the null mutation and the complementation of the different mutants, the expression of the corresponding genes in the respective mutants was assessed by RT-PCR and Southern blots (Supplemental Figure 4). Clearly, single mutants did not express the deleted gene and the double mutant failed to express both genes whereas the revertant strains showed the expression of the complemented genes (data not shown).

Phenotype of the Δcsm mutants

All stages of the biological cycle of single and double mutants of A. fumigatus were analysed to try to understand the role of the MMD-CHS in chitin synthesis.
a) Chitin synthase activity and cell wall chitin content of the mycelium

Previous studies have shown that the major chitin synthase activity in *A. fumigatus* was seen after proteolytic treatment of the mycelial membrane fractions (34). In these conditions, the overall chitin synthase activity quantified *in vitro* in mycelial extracts was not affected by the deletion of the *CSMA* or/and *CSMB* genes (Figure 2). Moreover, the chitin content of the mycelial cell wall (both 24 h and 36 h) was not statistically different in the single and double mutant strains compared to the parental strain (Figure 2). The apex and septa of the mycelium were labelled with calcofluor white at the same intensity in the parental and the mutant strains. In addition, deletion of the *CSMA* and/or *CSMB* genes was not compensated by an increase in the expression of other *CHS* genes as shown by the q-RT-PCR data (Supplementary Figure 5).

b) Mycelial growth

On agar media, there was a slight but significant reduction of the colony diameter among the mutant and parental strains when the medium was inoculated with 10-days old conidia (Figure 3A). In contrast, in liquid YG medium under shaken conditions, no difference in mycelial dry weight was seen among the mutant and parental strains (data not shown). After 24 h of growth, the mycelial morphology was similar in the parental and mutant strains except that the mycelia were significantly wider in the mutant strains (in µm, 1.2±0.2, 2.0±0.3, 1.6±0.2 and 1.9±0.3, respectively for the parental, ΔcsmA, ΔcsmB and ΔcsmA/ΔcsmB mutant strains). However, in the later stages (32-48 h) of growth, the differences in the mycelial morphology of the mutant and the parental strains was accentuated (in µm, 2.0±0.1, 3.3±0.2, 2.5±0.1 and 2.9±0.1, respectively for the parental, ΔcsmA, ΔcsmB and ΔcsmA/ΔcsmB mutant strains) and there was an intra-hyphal growth in the csm-mutants; the frequency of such intra-
hyphal growth was 70-80% in the ΔcsmA and ΔcsmA/ΔcsmB mutants and 10-20% in the ΔcsmB mutant (Figure 3B).

c) Conidiation

The colonies of ΔcsmA, ΔcsmB and ΔcsmA/ΔcsmB mutants were always white in the different agar media tested, in clear contrast to the greenish colour of the parental strain indicating an altered conidiation (Figure 3A). The reduction in the amount of conidia produced is shown in the Table 2. The Δcsm mutants sporulated very poorly with no more than 2% of the conidiation level of the parental strain. This conidiation defect resulted from the reduction in total number of conidiophores and also from the formation of morphologically altered heterogeneous conidiophores: ~10-15% of all the mutant conidiophores showed enlarged vesicles with less phialides and a reduced number of conidia (Figure 4). Incubation in media stabilized osmotically with KCl (or sucrose) restored only partially the conidiation capacity of the mutants (Table 2, Figure 3C).

d) Conidial germination

Although they had a similar size as the parental strain in the resting stage, the volume of the conidia increased significantly above parental strain during the course of germination. After 6 h of growth, their diameter was approximately 2-2.5-times larger than that of the parental strain (Figure 5A). However, these morphological changes did not affect the percentage of conidial germination. This increase in volume occurred in all the media tested and in the AMM it was accompanied by a slight delay in germination time of the Δcsm mutants. In addition, the use of different dyes indicated that the cell walls of the mutant conidia (10-days old) were more permeable than the parental strain. Intracellular labelling of 20-25% of the swollen conidia was seen after incubation of the conidia with 5 min in presence of calcofluor.
white (Figure 5B) or Trypan blue (0.4%) whereas there was no labelling of the parental strain morphotypes.

e) Conidial phenotype

In contrast to the mycelium, the ΔcsmA mutant showed a high reduction (>65%) in the amount of chitin in the conidial cell wall. The ΔcsmB and unexpectedly the ΔcsmA/ΔcsmB double mutant had levels of chitin similar to the parental strain. Moreover, the CSMA revertant in the ΔcsmA/ΔcsmB double mutant had chitin levels similar to the parental/single ΔcsmB mutant strains (Table 3) confirming that the reduction of chitin in the ΔcsmA mutant was specifically due to the CSMA deletion. Most importantly, the overall cell wall polysaccharide composition of all mutant strains was modified (Table 3). The decrease of the ratio AI/AS in the cell wall (due both to a reduction in the β-(1,3)-glucan and an increase in the α-(1,3)-glucan contents) indicated an alteration of the overall cell wall structural organisation. However, transmission electron microscopic (TEM) analysis of the mutant conidia did not show difference in the cell wall structure and width compared to that of the parental strain (data not shown). Like in the mycelium, qRT-PCR data did not show a difference in the expression of other CHS genes in the conidiating mycelia of the CSMA and CSMA/CSMB deletion mutants (Supplemental Figure 6). This result suggests that CSMA and CSMB controlled two different interconnected pathways associated to chitin synthesis, at least during conidial development.

High-resolution atomic force microscopic (AFM) imaging was used to gain insight into the surface ultra-structure of resting conidia (16, 37). As can be seen in Figure 6, high-resolution images revealed the presence of homogeneous layers of rodlets on the surface of the parental strain and ΔcsmB mutant. However, ΔcsmB mutant conidial surface showed amorphous
structure at places. In contrast, the surfaces of the ΔcsmA and ΔcsmA/ΔcsmB mutants were different of the parental strain, i.e. rodlet structures were either completely lacking or heterogeneous depending on the single cell that was analysed. The surface of most of these mutant conidia consisted essentially of smooth and/or granular amorphous structures (Figure 6A). These images are in apparent contrast with biochemical analyses of HF extraction of the conidia showing the presence of RodAp (the hydrophobic protein forming rodlet layer) in all the strains, though the amount of RodAp extracted from the ΔcsmA was less compared to that of the parental strain (Figure 6B). The presence of rodlet layer underneath the amorphous layer was confirmed by scratching the csm mutant conidial surface with the AFM probe (data not shown). Thus, the combination of the AFM and biochemical data provided direct evidence that rodlets were not missing in the ΔcsmA and ΔcsmA/ΔcsmB mutants but rather, they were overlaid by an amorphous layer.

Another proof of the cell wall disorganisation was the loss of viability of the conidia overtime. Beyond one-month storage conidia in the aerial condition at room temperature, conidia started to be inviable (Supplemental Figure 7). This loss of viability was exacerbated in the ΔcsmA/ΔcsmB double mutant. In addition, 90% of the conidia of the ΔcsmA and ΔcsmA/ΔcsmB mutant failed to germinate after storage at 4°C overnight in water (data not shown). The reduced survival of the conidia over time can lead to artificial differences seen in the size of the colony when the Petri-plates were inoculated with old conidia or conidia stored improperly (Supplemental Figure 7).

The *A. fumigatus* Δcsm mutants displayed an exquisite sensitivity to echinocandins

The sensitivity of the mutants to different well-known cell wall-disturbing compounds was tested on agar plates. Among the eight CHS mutants, only the csm mutants showed
hypersensitivity to caspofungin (Figure 7A/7B). The Δcsm (both single and double) mutants showed an exquisite hypersensitivity to all the echinocandins (caspofungin, micafungin and anidulafungin) which are specific inhibitors of the β-(1,3)-glucan synthesis in fungi (Supplemental Figure 8). After 24 h of incubation of the conidia with the drug, conidia of the Δcsm mutants were abnormally enlarged and collapsed (Figure 7C). MIC calculated for all Δcsm mutants in the YG medium were 12 ng/ml and no paradoxical effect was seen with these mutants that were truly killed in vitro by echinocandins (Figure 7C). The lack of germ tube formation in the Δcsm mutants indicated that their resting conidia were susceptible to the echinocandins. In addition, pre-incubation of the mutant resting conidia and germinating conidia (grown either in the liquid Sabouraud medium (8 h at 37°C) or in the RPMI-MOPS medium (12 h at 37°C) for 1 h in the caspofungin solution (0.25 μg/ml) resulted in the death of both the morphotypes (as determined by the rezasurin method (10)), confirming that the cell wall structure of both conidium and hyphae was altered by the CSM deletion. In contrast, the Δcsm mutants were not more susceptible to nikkomycin Z compared to the parental strain. Susceptibility to drugs was indeed found to be specific to echinocandins since the Δcsm mutants were only slightly more sensitive to azoles, and not sensitive to calcofluor white and amphotericin B (Supplemental Figure 8).

To investigate if the increased susceptibility of the mutants to echinocandins was due to an alteration in β-(1,3)-glucan synthase activity, this enzymatic activity was measured in vitro using membrane preparations. No difference was found in the β-(1,3)-glucan synthase activity in the mutant strains as compared to the parental strain (data not shown). In addition, there was no difference in the mycelial β-(1,3)-glucan content of the ΔcsmB and ΔcsmA/ΔcsmB mutants compared to the parental strain, with only a very slight decrease in the ΔcsmA mutant (data not shown). These results suggest that the sensitivity of the Δcsm mutants to
echinocandins was not associated with a modification in the β-(1,3)-glucan synthase activity or the β-(1,3)-glucan content of the cell wall.

Discussion

Complexity of the chitin synthase family in filamentous fungi

Chitin synthases (CHS) belong to a very complex protein family in filamentous fungi and the precise biological function of the individual family members remain still poorly understood due to their insufficient biochemical characterization. To date, none of these transmembrane enzymes has been purified and enzymatic analysis still relies on the use of crude membrane preparation producing chitin from radio-labelled UDP-GlcNAc. Moreover, no direct correlation exists between the CHS activity measured in vitro using membrane preparations and the end product of the activity, the chitin content of the cell wall. One of the difficulties for understanding the role of CHS proteins is the fact that phenotypes of the mutants resulting from the deletion of orthologous genes in different fungal species are often very different (Supplementary Figure 9). For example, in S. cerevisiae, Cryptococcus neoformans (3) and Ustilago maydis (55), chitin synthase of class IV is responsible for the synthesis of most of the cell wall chitin. In contrast, studies in filamentous Ascomycetes like A. fumigatus or Neurospora crassa have not implicated the class IV, but the class III chitin synthases (CHSB of A. nidulans or CHSG of A. fumigatus) (15, 34). These comparative analyses have also shown that high levels of sequence similarities between two chitin synthases in the same species or taxonomically close species are not synonymous of similar biological function. For example, in A. fumigatus, two class III genes have been identified (CHSC and CHSG) and the double ΔchsC/ΔchsG mutant is not more affected in growth than the single ΔchsG mutant (34).
Role of the MMD-CHS in chitin synthesis

The data obtained here, showing the lack of difference in the chitin content of the mycelium of the parental and Δcsm mutant strains, was in agreement with previous data from Mellado and co-workers (34), but somehow contradicted another previous report (2); however, the small decrease in chitin synthesis reported for the ΔchsE (csmA) mutant in the Aufauvre-Brown and co-worker’s study is technically questionable based on the methodology used by them (enzymatic method, efficiency of which is influenced by the structural organisation of the cell wall, in contrast to the chemical hydrolysis method used in the present study). These differences could also be due to the genetic background since the periodic hyphal swelling reported by Aufauvre-Brown and co-workers (2) using AF273 strain as the parental strain was not observed using the CBS144-89-ku80 background strain used in our study. Clearly, CSM-proteins are not responsible for the bulk of chitin synthesis in none of the fungal systems studied. However, specific analytical methods are still lacking to quantify the chitin defects in situ at the cellular level and the current chemical analysis are unable to reveal a difference in a specific location of chitin in the cell wall or in the modification of the types of chitin microfibrils associated to separate CHS genes as seen in C. albicans (26). Such analysis could be related to the effect of the CSM deletion on the reduction of the non-zymogenic activity (that accounted for less than 10% of the total chitin synthase activity). This activity quantified without trypsin at pH 8 and in presence of Co^{2+} and Ni^{2+} (22) was reduced in the single and double Δcsm mutants (data not shown), suggested that the differential effect of cations seen on chitin synthase activities in yeast (49) was also found in filamentous fungi.

Like in F. oxysporum, Fusarium verticilloides or Gibberella zeae, the double csm mutant of A. fumigatus is fully viable, whereas the double CSMA and CSMB gene deletion was synthetically lethal in A. nidulans (45), questioning whether it is an experimental artefact or a
biological adaptation of this species. The growth phenotype of the $\Delta_{csm}$ mutant in the different species analysed was also variable and go from a severely reduced growth in $G. zeae$ or $F. oxysporum$ mutants to a growth almost identical to the parental strain in $A. fumigatus$. The $\Delta_{csmA}$ mutant showed a decrease in the conidial cell wall chitin content. But unexpectedly, the deletion of $CSMB$ in the $csmA$ mutant restored a normal amount of chitin in the conidial cell wall. We were very cautious to verify that this result was really founded by constructing single and double $\Delta_{csm}$ mutant, for which we followed two different deletion strategies using different parental strains. It was also verified that the two genes AFUA_2G13430 and AFUA_2G13450 at the 3’ and 5’ end of the $CSMA$ and $CSMB$ operons were expressed showing that the deletion did not affect the neighbouring genes. Similarly and unexpectedly, the deletion of class V and VII chitin synthases of $F. oxysporum$ lead to a 40% increase in the cell wall chitin content compared to parental strain. We cannot explain presently the presence of wild-type level of chitin in the double $csm$ mutants of $A. fumigatus$ since this double deletion does not seem to be associated to compensatory expression of $CHS$ from other families (Supplemental Figures 5 and 6).

The present results show that $CSMA$ and $CSMB$ do not have overlapping functions and do not compensate for each other as already suggested by previous studies on $G. zeae$ or $F. verticilloides$. If the direct role of CSM proteins in the chitin synthesis has not been elucidated, the function of the MMD-domain in cellular trafficking of the chitin synthase has been extremely well dissected recently (42, 47). Using the model $U maydis$, Weber and co-workers showed that CHS with a myosin-17 motor domain is travelling along both central microtubules and peripheral filamentous actin (55). This transport is mediated by kinesin-1 and myosin-5; only a small percentage of the vesicles get exocystosed whereas the majority is
returned to the central core by the motor dynein. As shown earlier by the group of Horiuchi (19), successful exocytosis at the hyphal tip requires the MMD.

Chitin synthesis in vegetative hyphae and aerial structures uses two different pathways

In *A. fumigatus*, the major morphological perturbations resulting from the MMD-CHS deletion was associated to conidiogenesis. Single and double Δcsm mutants of *A. fumigatus* sporulated poorly due to the formation of abnormal conidiophores that contained very few conidia, whereas conidiation was normal in all the other chitin synthase mutants, suggesting that chitin is essential for the maintenance of the conidiophores in an aerial position and for the production of conidia. The role of CSM proteins in the production of aerial hyphae, appressorium formation, conidiogenesis and sexual reproduction has been documented already in other filamentous species (23, 24, 32, 46). In spite of this phenotype, the amount of chitin in the conidium and conidiating structures or appressorium has not been investigated previously in other fungal species. These data suggest that CSM-proteins are more important for hyphal specialisation than for development. However, the specific role of individual chitin synthases in the construction of the conidial cell wall remains unknown.

The deletion of CSM-CHS impacts on *A. fumigatus* susceptibility to antifungal drugs

Even though CSM deletions did not modify the amount of chitin in the mycelial cell wall, they altered the cell wall structural organisation. In case of the Δcsm mutant, modifications in the cell wall polysaccharides that were associated to a loss of viability and permeability changes, could be responsible for an abnormal swelling of conidia during germination and an increased drug uptake. These modifications should be very peculiar to promote the susceptibility of the Δcsm mutants to echinocandins and not to other drugs. The chemical nature of the structural modifications as well as the mechanisms altering the cell wall
permeability should be investigated because they could lead to the discovery of new antifungal targets.

The increased sensitivity of the Δcsm mutants to caspofungin was in agreement with the synergistic antifungal effect of a combination of a chitin synthesis inhibitor such as nikkomycin and a β-(1,3)-glucan synthase inhibitor such as caspofungin. However, this increase in the sensitivity to echinocandins is only seen with the class V and VII mutants whereas other single CHS (A, B, C and D) deletion mutants have sensitivity to echinocandins similar to the parental strain. It is well known that modifications of the cell wall chitin are associated to a modification of the susceptibility to echinocandins (52). In A. fumigatus, compared to the parental strain, calcineurin mutants contain lower amounts of β-glucan and chitin in the presence of caspofungin and are more sensitive to this drug (17). In contrast, though the Δras mutant contains a lower amount of β-glucans, it is more resistant to caspofungin due to an increase in the cell wall chitin content (17). Similarly, in C. albicans, an increase of cell wall chitin consecutive to the growth in the presence of calcium and calcofluor white is also associated to a reduction in the sensitivity to caspofungin (53). However, Δpmt2 mutant are also extremely sensitive to echinocandins without any modifications of the chitin levels (36). These data suggested that echinocandin influx is controlled by several mechanisms, which are yet to be investigated.

Looking at the chitin synthase family in A. fumigatus is like working on a giant puzzle, where many pieces are still missing. Results obtained to-date show that the biochemical and cellular functions of each CHS genes as well as the interactions and compensatory effects among all CHS-proteins are extremely complex and remain poorly understood. Therefore the acquisition
of new bits of information is absolutely required to assemble the full scenario for chitin
synthesis in filamentous ascomycete biology.

Acknowledgements:

Work in the Aspergillus unit was partly supported by European grants Fungwall, Antifun and
ESF Fuminomics; Work at the Université catholique de Louvain was supported by the
National Foundation for Scientific Research (FNRS), the Université catholique de Louvain
(Fonds Spéciaux de Recherche), the Région Wallonne, the Federal Office for Scientific,
Technical and Cultural Affairs (Interuniversity Poles of Attraction Programme), and the
Research Department of the Communauté française de Belgique (Concerted Research
Action). Y.F.D. and D.A. are Senior Research Associate and Post-doctoral Research Fellow
of the FRS-FNRS. Research at CR’s lab was supported by CICYT grants BIO2007-60779 and
BFU2010-18632.


### Table 1: Strains used in this study

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<th>Strain</th>
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<th>Source</th>
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<td>CEA17</td>
<td>CBS 144.89 pyrG (Auxotrophic pyrG1)</td>
<td>(12)</td>
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<tr>
<td>AF14</td>
<td>CEA17 pyrG1+ (prototrophic wild-type; isogenic of CEA17)</td>
<td>(51)</td>
</tr>
<tr>
<td>CBS 144.89</td>
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<td>(46)</td>
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<td>CEA17ΔakuB3486</td>
<td>CEA17ΔakuB3486</td>
<td>(13)</td>
</tr>
<tr>
<td>A30</td>
<td>CEA17ΔcsmA::hisG-pyrG-hisG</td>
<td>This study</td>
</tr>
<tr>
<td>B1</td>
<td>CEA17ΔcsmB::hisG-pyrG-hisG</td>
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</tr>
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<td>D78</td>
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<td>A30pyrG-</td>
<td>CEA17ΔcsmA::hisG</td>
<td>This study</td>
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<td>ΔcsmA</td>
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<td>ΔcsmAΔcsmB</td>
<td>CEA17ΔakuB3486ΔcsmA::six/ΔcsmB::six-β-rec-hygroR-six</td>
<td>This study</td>
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Table 2: Conidiation of the ΔcsmA, ΔcsmB, ΔcsmA/ΔcsmB mutants and the parental strain in presence or absence of KCl (Mean ± SD)

<table>
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<tr>
<th>Strain</th>
<th>Malt agar 2%</th>
<th>Malt agar + KCl, 6%</th>
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<tr>
<td>Parental</td>
<td>7.8x10⁸ ± 0.72</td>
<td>4.4x10⁸ ± 0.53</td>
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<tr>
<td>ΔcsmA</td>
<td>3.53x10⁵ ± 0.31</td>
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<tr>
<td>ΔcsmB</td>
<td>1.33x10⁷ ± 0.31</td>
<td>3.2x10⁷ ± 0.2</td>
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<tr>
<td>ΔcsmA/ΔcsmB</td>
<td>1.53x10⁷ ± 0.31</td>
<td>4.73x10⁷ ± 0.61</td>
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Table 3: Monosaccharide composition of the parental, mutant and revertant conidial cell wall

(Mean ± SD)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Alkali-insoluble (AI) fraction</th>
<th>Alkali-soluble (AS) fraction</th>
<th>AI/AS</th>
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<tr>
<td></td>
<td>Man</td>
<td>Glu</td>
<td>Gal</td>
</tr>
<tr>
<td>WT</td>
<td>8.6±0.6</td>
<td>53.6±0.9</td>
<td>2.9±0.4</td>
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<td>ΔcsmA</td>
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<td>42.6±1.7</td>
<td>1.4±0.4</td>
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<td>ΔcsmB</td>
<td>4.6±1.0</td>
<td>43.6±0.9</td>
<td>2.5±0.8</td>
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<td>ΔcsmA/ΔcsmB</td>
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<td>2.9±0.4</td>
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<td>ΔcsmA::CSMA</td>
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<td>53.4±1.7</td>
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<td>ΔcsmB::CSMB</td>
<td>7.8±0.4</td>
<td>50.9±0.4</td>
<td>3.6±1.0</td>
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<tr>
<td>ΔcsmA/ΔcsmB::CSMA</td>
<td>3.0±0.3</td>
<td>43.4±0.2</td>
<td>1.9±0.1</td>
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<tr>
<td>ΔcsmA/ΔcsmB::CSMB</td>
<td>3.6±0.9</td>
<td>44.4±2.7</td>
<td>2.6±0.6</td>
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</table>

Man-Mannose; Glu-Glucose; Gal-Galactose; GlcN - Glucosamine
Figure 1: (A) Organisation of the CSMA and CSMB genes in a head-to-head configuration in *A. fumigatus* Chromosome 2. The orientation of transcription is shown by arrowheads. The distance between both translational start points is 3221 bp; (B) Structures of CsmA and CsmB proteins. The myosin motor-like domains (MMD) and the chitin synthase domains (CSD) are indicated by solid black and grey boxes respectively. The P-loop, Switch I and Switch II motifs are also shown.
Figure 2: Mycelial chitin content (% in the cell wall, after 24 h growth; mean ± SD of four individual experiments) and zymogenic chitin synthase (CS; mean ± SD of three different experiments) activity of the parental and the single and double Δcsm mutants.
Figure 3: Colony growth of the parental and Δcsm mutant strains in rich media without (A) and with (C) an osmotic stabilizer (KCl) after 48 h at 37°C; (B) Calcofluor white staining of the parental and the Δcsm mutant strains’ mycelia after 32 h growth in liquid Sabouraud culture medium at 37°C.
Figure 4: Morphology of the abnormal conidiophores of the ΔcsmA mutant strains after 4-days of growth on malt (2%) + KCl (6%) agar media at 37°C. Note in ΔcsmB, of the two conidiophores, one is similar to that of the parental strain.
Figure 5: (A) Conidial germination of the parental and the Δcsm mutant strains showing that the diameters of the mutant conidia are larger (grown in the liquid Sabouraud culture medium at 37°C for 7½ h); (B) Calcofluor white staining of swollen conidia of the parental and the Δcsm mutant strains (grown in the liquid Sabouraud culture medium at 37°C for 4½ h) showing the permeability of swollen conidia to the dye only in the Δcsm mutants.
Figure 6: (A) AFM deflection images in buffer of *A. fumigatus* conidia of the parental strain and ΔcsmB mutant showing the presence of rodlet layer on its surface; ΔcsmB mutant shows amorphous layer at places. AFM deflection images of *A. fumigatus* conidia of ΔcsmA mutant and ΔcsmA/ΔcsmB double mutant revealing amorphous layers devoid of rodlets. For every strain, the images shown are representative of at least 10 conidia; (B) HF extracts from the ΔcsmA, ΔcsmB, ΔcsmA/ΔcsmB and parental strain conidia showing the presence of RodAp (conidial surface protein, hydrophobin, responsible for the rodlet structure) in all the strains.
Figure 7: (A) YG Plates containing 0.1 µg/ml of caspofungin inoculated with serial 10-fold dilutions of conidia (2x10^6) from all single Δchs mutants of A. fumigatus (with the exception of ΔcsmB mutant); (B) Ten-fold conidial dilutions (2x10^6) of ΔcsmA, ΔcsmB, ΔcsmA/ΔcsmB and parental strains were spotted on YG plates with 0.1 µg and 6 ng/ml of caspofungin. Plates were incubated 3-days at 28°C; (C) Microscopic analyses of the conidia abnormally swollen on the plates containing 0.1 µg caspofungin at 37°C for 24 h.