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Chitin Synthases with a Myosin Motor-Like Domain Control the Resistance of *Aspergillus fumigatus* to Echinocandins

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Aspergillus fumigatus **has two chitin synthases (*CSMA* and *CSMB*) 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* mutant strains were constructed and analyzed. 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 of the mutants and the parental strain. In the conidia, chitin content in the Δ*csmA* strain cell wall was less than half the amount found in the parental strain. In contrast, the Δ*csmB* mutant strain and, unexpectedly, the Δ*csmA*/Δ*csmB* mutant strain did not show any modification of chitin content in their conidial cell walls. In contrast to the hydrophobic conidia of the parental strain, conidia of all of the *csm* mutants were hydrophilic due to the presence of an amorphous material covering the hydrophobic surface-rodlet layer. The deletion of *CSM* genes also resulted 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 disorganization of the cell wall structure, even though they contribute only weakly to the overall cell wall chitin synthesis.**

Chitin, a microfibrillar homopolymer of β-(1,4)-linked *N*-acetylglucosamine (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 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 sequences of their conserved regions, CHS proteins have been classified into two families that include seven classes (see Fig. S1 in the supplemental material) (38, 41). Family I includes classes I to III, while classes IV to VII belong to Family II (9, 55). The 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 1 (class III in *Encephalitozoon cuniculi*) to 3 (*Saccharomyces cerevisiae*, classes I, II, and IV) to >10 in filamentous fungi encompassing all of the *CHS* classes, with 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 play a role in hyphal growth. The function of the different *CHS* genes is only understood in *S. cerevisiae*, where it has been shown that each class of CHS proteins has a 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 remain poorly understood. It is especially puzzling to see that some chitin synthase mutants, such as *chs1* of *Neurospora crassa* or *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 normal chitin synthesis activity *in vitro* (15, 54).

Eight chitin synthase genes were identified in *Aspergillus fumigatus* (*CHSA*, *CHSB*, *CHSC*, *CHSD*, *CHSG*, *CHSF*, *CSMA* [earlier called *CHSE*], and *CSMB*). To date, six simple and three double mutants were constructed (G/C, A/C, and G/E) (32–35, 40). Inactivation of the Family I genes, *CHSA* (class I), *CHSB* (class II), and *CHSC* and *CHSG* (class III), individually did not lead to any growth phenotype, with the exception of *CHSG*. Characterization of Family II *CHS* remained incomplete in *A. fumigatus*; only *CSMA* (class V) and *CHSD* (class VI) were analyzed (32). Disrup-

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tion of *CSMA* (class V) leads to an altered growth phenotype (poor conidiation, reduction in the colony radial growth rate, and decrease in 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 organization of the cell wall chitin (20, 21, 24, 27, 29, 55, 56). To date it has been shown only that these enzymes have a conserved myosin-17 myosin motor-like domain (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 analyzed. 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 chitin synthesis in the conidial cell wall. Even though the deletion of *CSMA* and *CSMB* was not associated with a modification of the mycelial chitin level, the overall organization of the cell wall polysaccharides was perturbed, leading to an increased sensitivity to antifungal drugs.

MATERIALS AND METHODS

Strains and culture conditions. *Aspergillus fumigatus* strains used in this work are listed in Table S1 in the supplemental material. 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 medium to enhance conidiation. Conidia were collected from agar medium 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. For strategy 1, plasmids pCJ-E4, pCJ-Eb4, and pCJ-D2 were used for the construction of the Δ*csmA*, Δ*csmB*, and Δ*csmA*/Δ*csmB* mutants, respectively. These plasmids carry a DNA fragment designed to delete most of the open reading frames (ORFs) of the *CSMA* and *CSMB* genes. The strategy outlined in Fig. S2 in the supplemental material was used to produce strains with nonfunctional, disrupted *A. fumigatus CSM* (*AfCSM*) genes. The knockout Δ*csm* plasmids (pCJ-E4, pCJ-Eb4, and pCJ-D2) were constructed in the pGEMT 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 Table S1 in the supplemental material). 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 NotI digestion. The construction of the Δ*csmB* 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 NotI digestion. For the construction of the double mutant Δ*csmA*/Δ*csmB* strain, 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 NotI digestion. The transformation of *A. fumigatus* strain CEA17 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 analyzed 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 previously (51). Integration of the cassette at the correct locus was confirmed by Southern blotting with genomic DNA digested by XmnI for the Δ*csmA* strain, NcoI for the Δ*csmB* strain, and BglII for the double mutant strain (see Fig. S2).

Due to unexpected results with the Δ*csmA*/Δ*csmB* double mutant, we decided to undertake another replacement strategy in a different genetic background, termed strategy 2. The Δ*csmA*/Δ*csmB* double deletion mutant was constructed in the CEA17_Δ*akuB*^{KU80} 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 system (Life Technologies, Carlsbad, CA), 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 6,796 bp for *csmA* and 6,806 bp for *cmsB* were released from the resulting vector via EcoRV and FspI, respectively. The CEA17Δ*akuB*^{KU80} parental strain was transformed with the *csmA* replacement cassette by electroporation. Transformants obtained were analyzed by diagnostic PCR with oligonucleotides Forw *csmA* and Sv630 (see Table S1 in the supplemental material). The Δ*csmA* deletion mutant obtained was cultivated in the 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 Δ*csmA*x strain were then verified by Southern blot analysis (see Fig. S3). To obtain the Δ*csmA*/Δ*csmB* double deletion mutant, the *csmB* replacement cassette was transformed in the Δ*csmA*x recipient strain. Transformants obtained were analyzed by diagnostic PCR with oligonucleotides Forw *csmB* and Sv630 and Southern blot analysis (see Table S1).

Construction of revertant strains. Complementation of the Δ*csm* mutants was obtained by a transformation strategy using a wild-type (WT) copy of the genes in the recombinant plasmid pPYRGQ3, which contains the *pyrG* resistance 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 Table S1 in the supplemental material. ORFs of the genes *CSMA* and *CSMB* were amplified from genomic DNA of the CEA17 *pyrG1*$^{+}$ (AF14) strain and inserted into pPYRGQ3 that had been digested with XbaI and XmaI to yield pPYRQ3_*CSMA* and pPYRQ3_*CSMB*. These plasmids were linearized with NotI and transformed into the corresponding *pyrG*$^{-}$ strains of *A. fumigatus*. The presence of the WT copy of the genes was confirmed by PCR and Southern blotting using the probes amplified with the primers sE_F/sE_R for *CSMA* complementation and sEb_F/sEb_R for *CSMB* complementation (see Fig. S4).

DNA isolation and hybridization. DNA was isolated from *A. fumigatus* using the extraction procedure described by Calera and coworkers (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 by following standard protocols. Probes for *AfCSMA* and *AfCSMB* were obtained by restriction enzyme digestion of the appropriate clones. The Rediprime Random Prime labeling system kit (Amersham) was used to label DNA probes according to the manufacturer's instructions.

Real-time reverse transcription-PCR (RT-PCR). Vegetative mycelium was obtained after 16 h of culture in a glucose (3%)-yeast extract (YE; 1%) liquid medium. Mycelial growth (for 24 h) on malt agar covered with a cellophane membrane (DryEase cellophane; Invitrogen) corresponded to the onset of conidiating morphotype. Fungal material was 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 minikit. Quantita-

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tive PCR assays were performed as described in Mouyna et al. (36). The expression ratios were normalized to *TEF1* expression and calculated according to the $2^{\Delta CT}$ 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* to *CHSG*) has been analyzed. Primers used for these genes and the gene accession numbers are given in Table S2 in the supplemental material.

Antifungal assays. Ten-fold dilutions, starting at 2×10^6 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-morpholinepropanesulfonic acid (MOPS) liquid medium using the resazurin method (10). The effect of nikkomycin Z was tested by the CLSI M38-A2 protocol and the resazurin method (10).

GS and CHS activity assays. β-(1,3)-Glucan synthase (GS) activity was measured according to a previously described procedure using an excess of GTPγS (4). CHS activity measurements were carried out with crude membrane extracts obtained as follows: flasks with 200 ml of Sabouraud medium were inoculated with 5×10^5 conidia per ml of the different strains and incubated for 17 h at 37°C in an orbital incubator. Mycelia were collected by filtration under vacuum, washed with water, and disrupted in an 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 ($8,000 \times g$, 10 min), and the supernatant was centrifuged for 35 min at $100,000 \times g$. Membranes were then washed in 50 mM Tris-HCl (pH 7.5) containing 5 mM magnesium acetate, centrifuged again for 35 min at $100,000 \times g$, resuspended in the same buffer containing 30% glycerol, and stored at −80°C. CHS activity was measured by the incorporation of UDP-*N*-acetylglucosamine (UDP-GlcNAc) into chitin with trypsin treatment using a modification of the protocol described by Choi and Cabib (8). The assay system for CHS activity consisted of 50 μg of membranes, 32 mM GlcNAc, 1.1 mM UDP-[^{14}C]GlcNAc (293 mCi mmol^{-1}; Amersham Life Science), and 4.3 mM magnesium acetate in 30 mM morpholineethanesulfonic acid (MES; pH 6.5) in a total volume of 50 μl. For the proteolytic activation step, 2 μl of trypsin (1 to 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 h per mg of protein.

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

AFM. Atomic force microscopy (AFM) images were obtained in contact mode at room temperature (20°C) in phosphate-buffered saline (PBS) using a Nanoscope V Multimode atomic force microscope with oxide-sharpened microfabricated Si3N4 cantilevers with 0.01 N/m spring constants (Bruker, Santa Barbara, CA). For live cell imaging, conidia were immobilized by mechanical trapping into polycarbonate porous membranes (3-μm diameter; It4ip). After filtering a cell suspension (200 μl; 2.5×10^6 cells/ml), the filter was carefully rinsed in PBS, cut (1 by 1 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 (12).

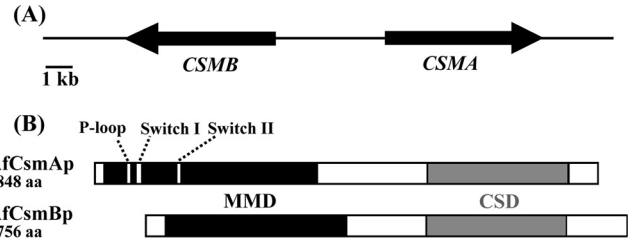

FIG 1 (A) Organization of the *CSMA* and *CSMB* genes in a head-to-head configuration in *A. fumigatus* chromosome 2. The orientation of transcription is shown by arrowheads. The distance between both translational start points is 3,221 bp. (B) Structures of CsmA and CsmB proteins. The myosin motor-like domains (MMD) and the chitin synthase domains (CSD) are indicated by solid black and gray boxes, respectively. The P-loop, Switch I, and Switch II motifs are also shown.

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 (the number of hyphae measured for width was 30), was performed after staining of the mycelium with calcofluor white (aqueous solution at a final concentration of 5 μg/ml). Resting and germinating conidia were stained with Trypan blue and calcofluor white (0.4% and 5 μg/ml, respectively, for 5 min). The percentage 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 N_2 and reconstituted in H_2O (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, 10^4/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 a hemocytometer. The percentage of germination was quantified after spotting 5×10^3 conidia on Sabouraud agar medium or PDA spread over the glass slides, incubating at 37°C, and counting the germ tubes microscopically every 1 h between 7 and 12 h.

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

RESULTS

Sequence analysis of the two *CHS* genes with an 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 (MMD) were located nearby in chromosome 2 of *A. fumigatus*. For better homology to the *A. nidulans* nomenclature, the genes AFUA_2G13440 (*CHSE*) and AFUA_2G13430 were named *AfCSMA* and *AfCSMB*, respectively (*viz.*, chitin synthase with 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 3,221 bp between their translational start points (Fig. 1). The analysis of the DNA sequence of the *CSMB* gene revealed an ORF of 5,446 bp encoding a 1,756-amino-acid polypeptide organized in four exons interrupted by three introns. Originally described as *CHSE* (2), the full length of the *CSMA* gene was 5,740 bp and encoded a protein with 1,848 amino acids. The proteins encoded by *CSMA* and *CSMB*

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TABLE 1 Strains used in this study

Strain	Genotype	Source
CEA17	CBS 144.89 *pyrG*$^{-}$ (auxotrophic *pyrG1*)	14
AF14	CEA17 *pyrG1*$^{+}$ (prototrophic wild type; isogenic CEA17)	51
CBS 144.89	WT	46
CEA17_Δ*akuB*^{KU80}	CEA17 Δ*akuB*^{KU80}	13
A30	CEA17 Δ*csmA*::*hisG-pyrG-hisG*	This study
B1	CEA17 Δ*csmB*::*hisG-pyrG-hisG*	This study
D78	CEA17 Δ*csmA* Δ*csmB*::*hisG-pyrG-hisG*	This study
A30 pyrG^{-}	CEA17 Δ*csmA*::*hisG*	This study
B1 pyrG^{-}	CEA17 Δ*csmB*::*hisG*	This study
D78 pyrG^{-}	CEA17 Δ*csmA* Δ*csmB*::*hisG*	This study
R1	CEA17 Δ*csmA*::*hisG csmA*::*pyrG*	This study
S5	CEA17 Δ*csmB*::*hisG csmB*::*pyrG*	This study
pA1	CEA17 Δ*csmA* Δ*csmB*::*hisG csmA*::*pyrG*	This study
pB1	CEA17 Δ*csmA* Δ*csmB*::*hisG csmB*::*pyrG*	This study
Δ*csmA*	CEA17_Δ*akuB*^{KU80} Δ*csmA*::*six*-β-*rec-hygroR-six*	This study
Δ*csmA*x	CEA17_Δ*akuB*^{KU80} Δ*csmA*::*six*	This study
Δ*csmA* Δ*csmB*	CEA17_Δ*akuB*^{KU80} Δ*csmA*::*six* Δ*csmB*::*six*-β-*rec-hygroR-six*	This study

genes showed an overall similarity of only 25% but presented Family II chitin synthase domains (CSD) at their C-terminal ends that displayed 60% similarity between them. In their N-terminal ends, the CsmA and CsmB proteins had an 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 (see Fig. S2 and S3 in the supplemental material). 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 blotting. All of the mutants and the revertants constructed are shown in Table 1. 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 blotting (see Fig. S4). 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 Δ*csm* mutants. All stages of the biological cycle of single and double mutants of *A. fumigatus* were analyzed to try to understand the role of MMD-*CHS* in chitin synthesis.

(i) 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). Under these conditions, the overall chitin synthase activity quantified *in vitro* in mycelial extracts was not affected by the deletion of the *CSMA* and/or *CSMB* genes (Fig. 2). Moreover, the chitin content of the mycelial cell wall (at both 24 and 36 h) was not statistically different in the single and double mutant strains compared to the parental strain (Fig. 2). The apex and septa of the mycelium were labeled with calcofluor white at the same intensity in the parental and mutant strains. In addition, deletion of the *CSMA* and/or *CSMB* genes was not compensated for by an increase in the expression of other *CHS* genes, as shown by the quantitative RT-PCR (qRT-PCR) data (see Fig. S5 in the supplemental material).

(ii) 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-day-old conidia (Fig. 3A). In contrast, in liquid YG medium under shaking, 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 for the parental and Δ*csmA*, Δ*csmB*, and Δ*csmA*/Δ*csmB* mutant strains, respectively). However, in the later stages (32 to 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 for the parental and Δ*csmA*, Δ*csmB*, and Δ*csmA*/Δ*csmB* mutant strains, respectively), and there was an intrahyphal growth in the *csm* mutants; the frequency of such intrahyphal growth was 70 to 80%

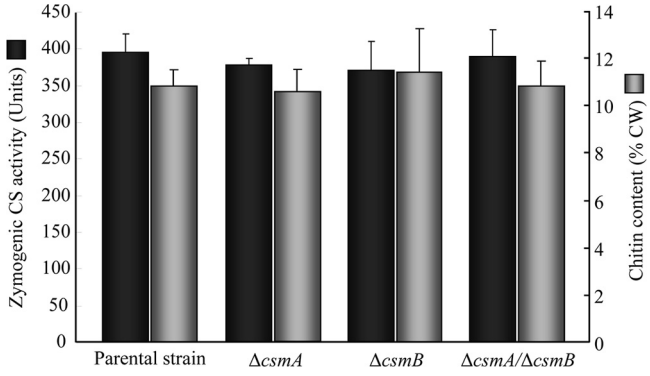

FIG 2 Mycelial chitin content (percent in the cell wall [CW] after 24 h of growth; data are means ± standard deviations [SD] from four individual experiments) and zymogenic chitin synthase (CS; means ± SD from three different experiments) activity of the parental strain and the single and double Δ*csm* mutant strains.

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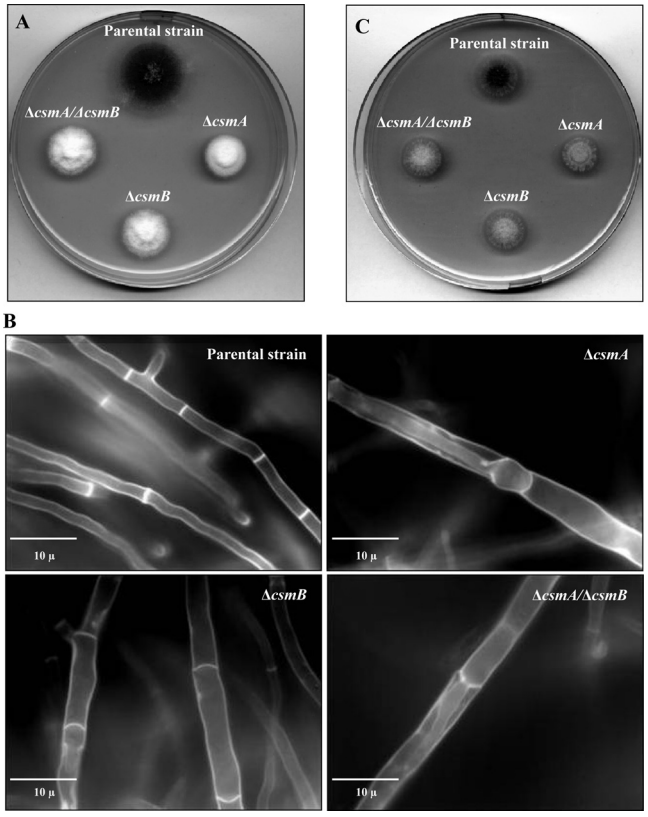

FIG 3 Colony growth of the parental and Δ*csm* mutant strains in rich media without (A) and with (C) an osmotic stabilizer (KCl) after 48 h at 37°C. (B) Calcofluor white staining of the parental and the Δ*csm* mutant strains' mycelia after 32 h of growth in liquid Sabouraud culture medium at 37°C.

in the Δ*csmA* and Δ*csmA*/Δ*csmB* mutants and 10 to 20% in the Δ*csmB* mutant (Fig. 3B).

(iii) Conidiation. The colonies of Δ*csmA*, Δ*csmB*, and Δ*csmA*/Δ*csmB* mutant strains were always white in the different agar media tested, in clear contrast to the greenish color of the parental strain, indicating an altered conidiation (Fig. 3A). The reduction in the amount of conidia produced is shown in Table 2. The Δ*csm* 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 numbers of conidiophores and also from the formation of morphologically altered heterogeneous conidiophores: ~10 to 15% of all of the mutant conidiophores showed enlarged vesicles with fewer phialides and a reduced number of conidia (Fig. 4). Incubation in media stabilized osmotically with KCl (or sucrose) only partially restored the conidiation capacity of the mutants (Table 2 and Fig. 3C).

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

(v) Conidial phenotype. In contrast to the mycelium, the Δ*csmA* mutant showed a high reduction (>65%) in the amount of chitin in the conidial cell wall. The Δ*csmB* strain and, unexpectedly, the Δ*csmA*/Δ*csmB* double mutant had levels of chitin similar to those of the parental strain. Moreover, the *CSMA* revertant in the Δ*csmA*/Δ*csmB* double mutant had chitin levels similar to those of the parental strain and single Δ*csmB* mutant strain (Table 3), confirming that the reduction of chitin in the Δ*csmA* 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 AI/AS ratio in the cell wall [due both to a reduction in the β-(1,3)-glucan contents and an increase in the α-(1,3)-glucan contents] indicated an alteration of the overall cell wall structural organization. However, transmission electron microscopic (TEM) analysis of the mutant conidia did not show a difference in the cell wall structure and width compared to that of the parental strain (data not shown). As was the case for 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 (see Fig. S6 in the supplemental material). This result suggests that *CSMA* and *CSMB* controlled two different interconnected pathways associated with chitin synthesis, at least during conidial development.

High-resolution atomic force microscopic (AFM) imaging was used to gain insight into the surface ultrastructure of resting conidia (16, 37). As can be seen in Fig. 6, high-resolution images revealed the presence of homogeneous layers of rodlets on the surface of the parental strain and Δ*csmB* mutant. However, Δ*csmB* mutant conidial surfaces showed amorphous structures in places. In contrast, the surfaces of the Δ*csmA* and Δ*csmA*/Δ*csmB* mutant strains were different from those of the parental strain, i.e., rodlet structures were either completely lacking or heterogeneous depending on the single cell that was analyzed. The surface of most of these mutant conidia consisted essentially of smooth and/or granular amorphous structures (Fig. 6A). These images are in apparent contrast to biochemical analyses of HF extraction of the conidia showing the presence of RodAp (the hydrophobic protein forming the rodlet layer) in all of the strains, although the amount of RodAp extracted from the Δ*csm*A strain was less than that of the parental strain (Fig. 6B). The presence of a 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 evi-

TABLE 2 Conidiation of the Δ*csmA*, Δ*csmB*, Δ*csmA*/Δ*csmB* mutants and the parental strain in the presence or absence of KCl

Strain	Growth in[a]:	
	Malt agar (2%)	Malt agar + KCl (6%)
Parental	$(7.8 \times 10^8) \pm 0.72$	$(4.4 \times 10^8) \pm 0.53$
Δ*csmA*	$(3.53 \times 10^5) \pm 0.31$	$(1.2 \times 10^7) \pm 0.2$
Δ*csmB*	$(1.33 \times 10^7) \pm 0.31$	$(3.2 \times 10^7) \pm 0.2$
Δ*csmA* Δ*csmB*	$(1.53 \times 10^7) \pm 0.31$	$(4.73 \times 10^7) \pm 0.61$

[a] Data are means ± SD.

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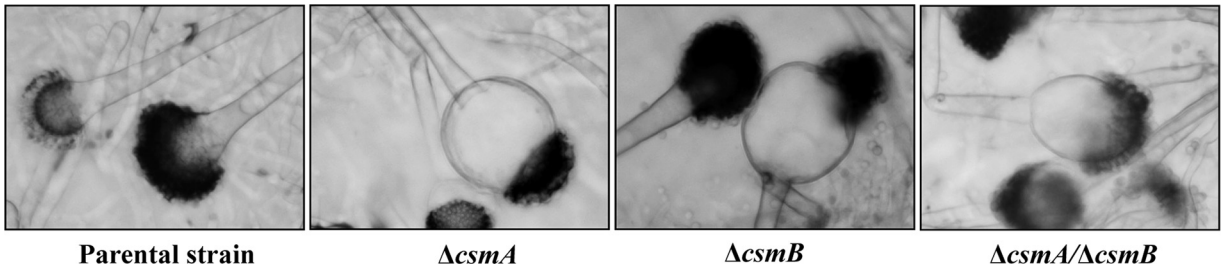

FIG 4 Morphology of the abnormal conidiophores of the Δ*csm* mutant strains after 4 days of growth on malt (2%) plus KCl (6%) agar medium at 37°C. Note that in the Δ*csmB* strain, of the two conidiophores, one is similar to that of the parental strain.

dence that rodlets were not missing from the Δ*csmA* and Δ*csmA*/Δ*csmB* mutants but rather were overlaid by an amorphous layer.

Another proof of the cell wall disorganization was the loss of viability of the conidia over time. Beyond 1 month of storage of conidia in aerial conditions at room temperature, conidia started to be inviable (see Fig. S7 in the supplemental material). This loss of viability was exacerbated in the Δ*csmA*/Δ*csmB* double mutant. In addition, 90% of the conidia of the Δ*csmA* and Δ*csmA*/Δ*csmB* mutant strains 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 are inoculated with old conidia or conidia stored improperly (see Fig. S7).

The *A. fumigatus* Δ*csm* 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 (Fig. 7A and B). The Δ*csm* (both single and double) mutants showed an exquisite hypersensitivity to all of the echinocandins (caspofungin, micafungin, and anidulafungin), which are specific inhibitors of the β-(1,3)-glucan synthesis in fungi (see Fig. S8 in the supplemental material). After 24 h of incubation of the conidia with the drug, conidia of the Δ*csm* mutants were abnormally enlarged and collapsed (Fig. 7C). MICs calculated for all Δ*csm* 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 (Fig. 7C). The lack of germ tube formation in the Δ*csm* mutants indicated that their resting conidia were susceptible to the echinocandins. In addition, preincubation of the mutant resting conidia and germinating conidia, grown either in liquid Sabouraud medium (8 h at 37°C) or in 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 morphotypes (as determined by the resazurin method [10]), confirming that the cell wall structures of both conidia and hyphae were altered by the *CSM* deletion. In contrast, the Δ*csm* mutants were not more susceptible to nikkomycin Z than the parental strain. Susceptibility to drugs was indeed found to be specific to echinocandins, since the Δ*csm* mutants were only slightly more sensitive to azoles and not sensitive to calcofluor white and amphotericin B (see Fig. S8 in the supplemental material).

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 compared to the parental strain (data not shown). In addition, there was no difference in the mycelial β-(1,3)-glucan content of the Δ*csmB* and Δ*csmA*/Δ*csmB* mutants compared to that of the parental strain, with only a very slight decrease in the Δ*csmA* mutant (data not shown). These results suggest that the sensitivity of the Δ*csm* 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.

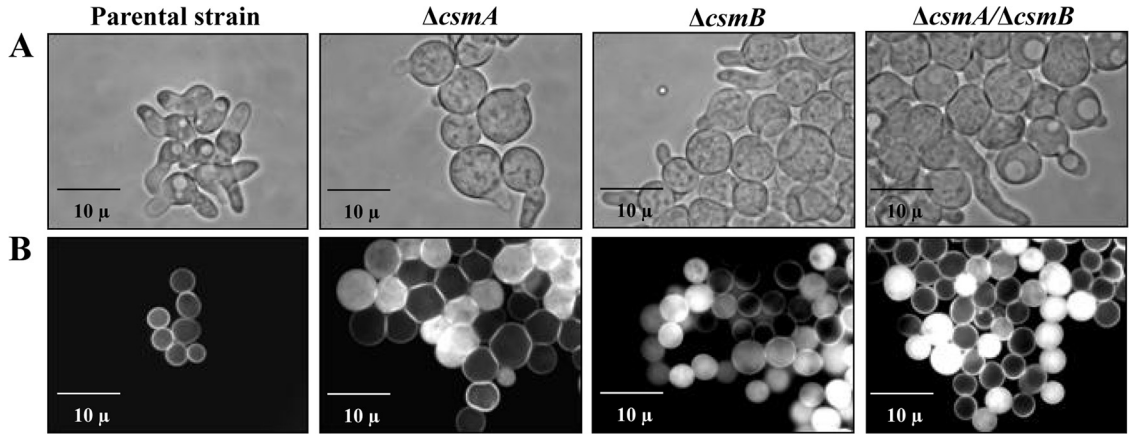

FIG 5 (A) Conidial germination of the parental and the Δ*csm* mutant strains showing that the diameters of the mutant conidia are larger (grown in liquid Sabouraud culture medium at 37°C for 7.5 h). (B) Calcofluor white staining of swollen conidia of the parental and the Δ*csm* mutant strains (grown in liquid Sabouraud culture medium at 37°C for 4.5 h) showing the permeability of swollen conidia to the dye only in the Δ*csm* mutants.

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TABLE 3 Monosaccharide composition of the parental, mutant, and revertant strain conidial cell walls[a]

Strain	Alkali-insoluble (AI) fraction				Alkali-soluble (AS) fraction			
	Man	Glu	Gal	GlcN	Man	Glu	Gal	AI/AS
WT	8.6 ± 0.6	53.6 ± 0.9	2.9 ± 0.4	4.1 ± 0.2	4.5 ± 0.5	24.4 ± 1.1	1.9 ± 0.2	2.25 ± 0.1
Δ*csmA*	3.8 ± 0.2	42.6 ± 1.7	1.4 ± 0.4	1.3 ± 0.1	6.0 ± 0.8	43.0 ± 1.0	2.0 ± 0.7	0.96 ± 0.1
Δ*csmB*	4.6 ± 1.0	43.6 ± 0.9	2.5 ± 0.8	3.5 ± 0.2	4.7 ± 0.9	39.6 ± 0.9	1.5 ± 0.5	1.18 ± 0.1
Δ*csmA* Δ*csmB*	4.3 ± 0.4	39.4 ± 0.9	2.9 ± 0.4	4.4 ± 0.2	5.6 ± 0.8	41.7 ± 1.4	1.8 ± 0.3	1.04 ± 0.0
Δ*csmA*::*CSMA*	7.5 ± 2.3	53.4 ± 1.7	2.8 ± 0.5	5.3 ± 0.5	5.2 ± 0.2	24.1 ± 1.6	1.7 ± 0.1	2.22 ± 0.2
Δ*csmB*::*CSMB*	7.8 ± 0.4	50.9 ± 0.4	3.6 ± 1.0	4.4 ± 0.2	4.6 ± 0.4	26.3 ± 0.1	2.5 ± 0.8	2.00 ± 0.1
Δ*csmA* Δ*csmB*::*CSMA*	3.0 ± 0.3	43.4 ± 0.2	1.9 ± 0.1	3.2 ± 0.2	4.0 ± 0.7	42.8 ± 0.8	1.6 ± 0.0	1.06 ± 0.0
Δ*csmA* Δ*csmB*::*CSMB*	3.6 ± 0.9	44.4 ± 2.7	2.6 ± 0.6	1.4 ± 0.2	5.4 ± 1.0	39.6 ± 0.8	3.0 ± 0.5	1.08 ± 0.1

[a] Data are means ± SD. Man, mannose; Glu, glucose; Gal, galactose; GlcN, glucosamine.

DISCUSSION

Complexity of the chitin synthase family in filamentous fungi. Chitin synthases belong to a very complex protein family in filamentous fungi, and the precise biological functions of the individual family members remain 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 radiolabeled 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 (see Fig. S9 in the supplemental material). 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 chitin synthases but have implicated those of class III (*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 with similar biological functions. For example, in *A. fumigatus*, two class III genes have been identified (*CHSC* and *CHSG*), and the Δ*chsC* Δ*chsG* double mutant is not more affected in growth than the Δ*chsG* single mutant (34).

Role of MMD-CS in chitin synthesis. The data obtained here, showing the lack of difference in chitin content of the mycelium of the parental and Δ*csm* mutant strains, was in agreement with previous data from Mellado and coworkers (34), but somehow it contradicted another previous report (2); however, the small decrease in chitin synthesis reported for the Δ*chsE* (*csmA*) mutant in the study of Aufauvre-Brown and coworkers (2) is technically questionable based on the methodology used by them (i.e., the enzymatic method, the efficiency of which is influenced by the structural organization of the cell wall, in contrast to the chemical hydrolysis method used in the present study). These differences also could be due to the genetic background, since the periodic hyphal swelling reported by Aufauvre-Brown and coworkers (2) using strain AF273 as the parental strain was not observed with the CBS144-89-*ku80* background strain used in our study. Clearly, CSM proteins are not responsible for the bulk of chitin synthesis in the fungal systems studied. However, specific analytical methods to quantify the chitin defects *in situ* at the cellular level are still lacking, and the current chemical analyses 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 with 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 nonzymogenic activity (which accounted for less than 10% of the total chitin synthase activity). This activity, quantified without trypsin at pH 8 and in the presence of Co^{+2} and Ni^{+2} (22), was reduced in the single and double Δ*csm* 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.

As in *F. oxysporum*, *Fusarium verticillioides*, or *Gibberella zeae*, the *csm* double mutant of *A. fumigatus* is fully viable, whereas the *CSMA* and *CSMB* double gene deletion was synthetically lethal in *A. nidulans* (45), questioning whether it is an experimental artifact or a biological adaptation of this species. The growth phenotype of the Δ*csm* mutant in the different species analyzed was also variable and goes from severely reduced growth in *G. zeae* or *F. oxysporum* mutants to growth almost identical to that of the parental strain in *A. fumigatus*. The Δ*csmA* mutant showed a decrease in the conidial cell wall chitin content. 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 well founded by constructing single and double Δ*csm* mutant strains, 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 neighboring genes. Similarly and unexpectedly, the deletion of class V and VII chitin synthases of *F. oxysporum* led to a 40% increase in the cell wall chitin content compared to that of the parental strain. We cannot explain presently the presence of a wild-type level of chitin in the double *csm* mutants of *A. fumigatus*, since this double deletion does not seem to be associated with compensatory expression of *CHS* from other families (see Fig. S5 and S6 in the supplemental material).

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 of *G. zeae* or *F. verticilloides*. Although the direct role of CSM proteins in chitin synthesis has not been elucidated, the function of the MMD in cellular trafficking of the chitin synthase has been extremely well dissected recently (42, 47). Using *U. maydis* as a model, Weber and coworkers

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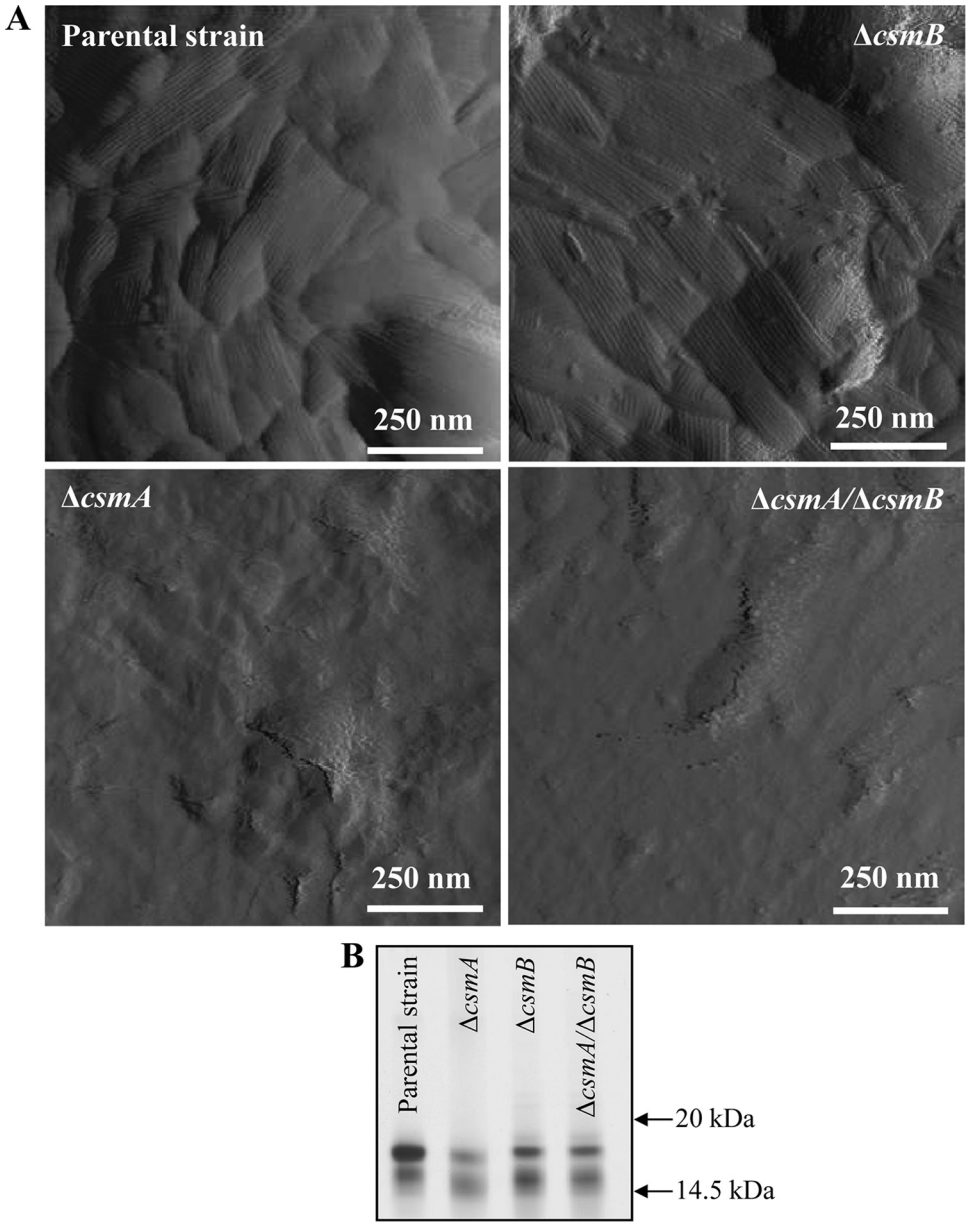

FIG 6 (A) AFM deflection images in buffer of *A. fumigatus* conidia of the parental strain and Δ*csmB* mutant showing the presence of a rodlet layer on its surface; the Δ*csmB* mutant shows an amorphous layer in places. AFM deflection images of *A. fumigatus* conidia of Δ*csmA* mutant and Δ*csmA*/Δ*csmB* double mutant strains revealing amorphous layers devoid of rodlets. For every strain, the images shown are representative of at least 10 conidia. (B) HF extracts from the Δ*csmA*, Δ*csmB*, and Δ*csmA* Δ*csmB* mutant and parental strain conidia showing the presence of RodAp (conidial surface protein, hydrophobin, responsible for the rodlet structure) in all of the strains.

showed that CHS with a myosin-17 motor domain travels 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 with conidiogenesis. Single and double Δ*csm* mutant strains of *A. fumigatus* sporulated poorly due to the formation of abnormal conidiophores that contained very few conidia, whereas conidiation was normal in all of 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

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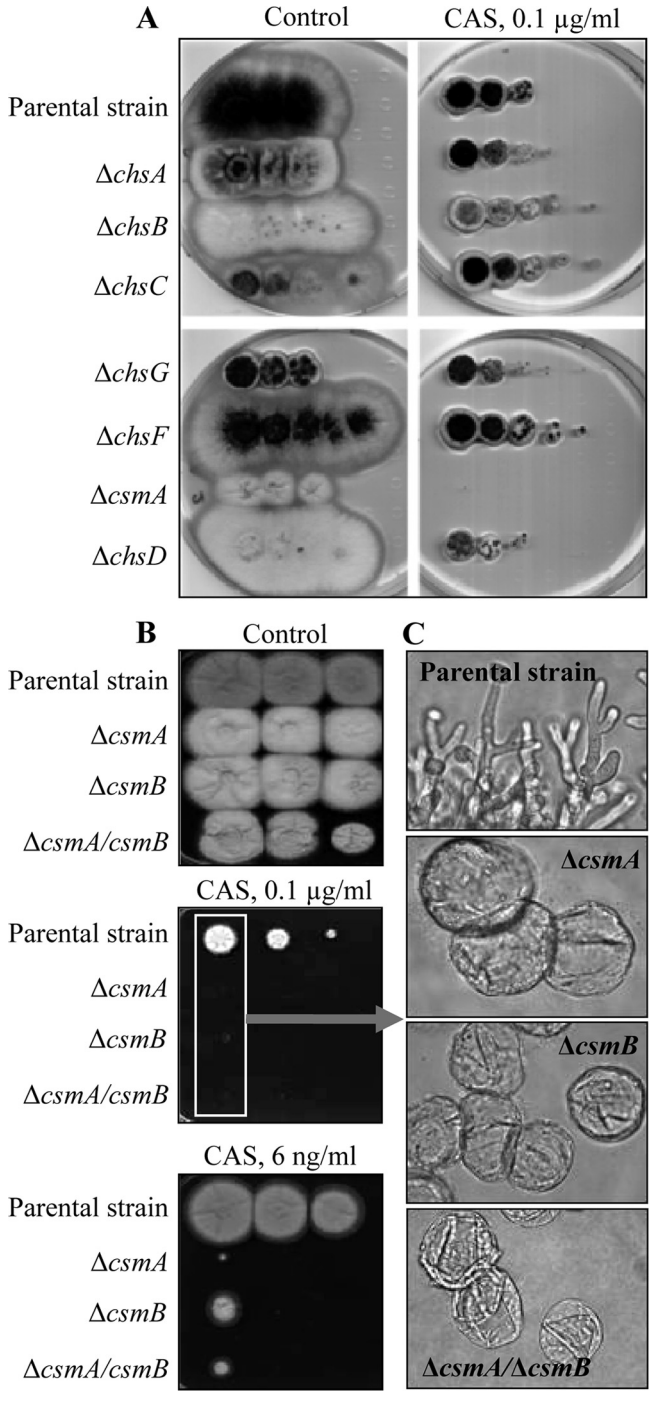

FIG 7 (A) YG plates containing 0.1 μg/ml of caspofungin inoculated with serial 10-fold dilutions of conidia (2×10^6) from all single Δ*chs* mutants of *A. fumigatus* (but not the Δ*csmB* mutant). (B) Ten-fold conidial dilutions (2×10^6) of Δ*csmA*, Δ*csmB*, and Δ*csmA* Δ*csmB* mutant strains and the parental strain were spotted on YG plates with 0.1 μg (6 ng/ml) caspofungin. Plates were incubated for 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.

suggest that CSM proteins are more important for hyphal specialization 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 affects *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 organization. In the case of the Δ*csm* mutant, modifications in the cell wall polysaccharides that were associated with a loss of viability and permeability changes could be responsible for an abnormal swelling of conidia during germination and an increased drug uptake. It would be very peculiar for these modifications to promote the susceptibility of the Δ*csm* 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 Δ*csm* 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* deletion mutant strains have sensitivity to echinocandins similar to that of the parental strain. It is well known that modifications of the cell wall chitin are associated with a modification of the susceptibility to echinocandins (52). In *A. fumigatus*, compared to the parental strain, calcineurin mutants contain smaller amounts of β-glucan and chitin in the presence of caspofungin and are more sensitive to this drug (17). In contrast, although the Δ*ras* mutant contains a smaller 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 with growth in the presence of calcium and calcofluor white is also associated with a reduction in the sensitivity to caspofungin (53). However, Δ*pmt2* mutant strains 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 have 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* gene 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.

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