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α1,3 Glucans Are Dispensable in *Aspergillus fumigatus*

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A triple α1,3 glucan synthase mutant of *Aspergillus fumigatus* obtained by successive deletions of the three α1,3 glucan synthase genes (*AGS1*, *AGS2*, and *AGS3*) has a cell wall devoid of α1,3 glucans. The lack of α1,3 glucans affects neither conidial germination nor mycelial vegetative growth and is compensated by an increase in β1,3 glucan and/or chitin content.

In *Aspergillus fumigatus*, α1,3 glucans are a major amorphous cell wall polysaccharide, accounting for 35 to 40% of the mycelial cell wall and 20 to 25% of the conidial cell wall (21). α1,3 glucans are also a major cell wall component of the yeast form of the human pathogens *Paracoccidioides brasiliensis*, *Histoplama capsulatum*, *Blastomyces dermatitidis*, and *Cryptococcus neoformans* and of the nonpathogenic model yeast *Schizosaccharomyces pombe* (14, 15, 24, 25, 27). In these species, the concentrations reported vary from 28% in *S. pombe* to 35 to 46% in the virulent yeast forms (13). In all pathogenic species studied to date, α1,3 glucans are important for virulence. In addition, in *S. pombe*, α1,3 glucans are essential for fungal viability. For *A. fumigatus*, it was shown that α1,3 glucans have a major adhesive role in the interactions between hyphae or germinating conidia (3, 8).

α1,3 glucans are synthesized by α1,3 glucan synthases, which are transmembrane enzymes with very high molecular masses (>200 kDa). The number of genes coding for Ags proteins varies between fungi. Only one *AGS* gene was found in *H. capsulatum* and *C. neoformans*, whereas five *AGS* genes were identified in *S. pombe* (14, 24, 25). In *A. fumigatus*, α1,3 glucans are synthesized by three α1,3 glucan synthases (Ags1p [AFUA_3G00910], Ags2p [AFUA_2G11270], and Ags3p [AFUA_1G15440]) (21). All three *A. fumigatus AGS* genes are expressed constitutively (21). Three single *AGS* mutants have been constructed in *A. fumigatus*. The *ags1*Δ mutant had a 50% reduction in the cell wall α1,3 glucan content of the mycelium (2). In spite of this cell wall defect, deletion of *AGS1* did not reduce the virulence of the strain in an experimental murine model of invasive aspergillosis (2). In contrast to the case for the *ags1*Δ mutant, the mycelial cell walls of *ags2*Δ and *ags3*Δ mutants had α1,3 glucan levels similar to that of the parental strain. In addition, no modification of the α1,3 glucan content of the conidial cell wall was seen in all single *ags*Δ mutants. Compensatory expression of the other members of the *AGS* family, which has been seen in all single *ags*Δ mutants, could explain the lack of a significant phenotype for each single *ags*Δ mutant (2, 21). For example, *AGS3* and *AGS2* showed increased expression in the *ags1*Δ mutant, and *AGS1* expression compensated for the lack of *AGS3* in the *ags3*Δ mutant (21).

Understanding the functional role of α1,3 glucans in *A. fumigatus* required the construction of a triple *ags1*Δ *ags2*Δ *ags3*Δ mutant devoid of α1,3 glucans. We report here the construction and growth phenotype of this triple mutant. As expected for the triple *AGS* deletion, the cell wall of the *ags1*Δ *ags2*Δ *ags3*Δ mutant did not contain any α1,3 glucans, but surprisingly, the mutant did not show any reduction in fungal viability and growth *in vitro*.

MATERIALS AND METHODS

Strains, plasmids, and culture conditions. The AkuB^{ku80} *pyrG*Δ strain was used as the parental strain (6). The AkuB^{ku80} *pyrG*Δ strain and mutants were maintained on 2% malt agar slants. Conidia were produced on 2% malt agar slants for 5 days at 37°C and recovered by vortexing with 0.05% (vol/vol) Tween 20 aqueous solution. For DNA extraction, mycelium was grown for 16 h at 37°C in a Sabouraud liquid medium supplemented with 10 mM uracil. For transformation experiments, complete medium supplemented with 100 μg/ml hygromycin (Sigma) was used for screening of the single mutant strain (*ags1*Δ::*HPH*), with 5 μg/ml phleomycin (Invitrogen) added for the double deletion mutant strain (*ags1*Δ::*HPH ags2*Δ::*BLE*), and minimal medium was used for the selection of the triple deletion mutant strain (*ags1*Δ::*HPH ags2*Δ::*BLE ags3*Δ::*PYRG*; named the *ags1*Δ *ags2*Δ *ags3*Δ strain in this study). For complementation, minimal medium supplemented with 50 μg/ml sulfonylurea (chlorimuron-ethyl; Sigma) or 10 μg/ml pyrithiamine (sigma) was used for screening of the transformants (10, 17, 29). For cell wall analysis, the mycelium was grown for 24 h at 37°C in Brian's medium (4). For nikkomycin Z (Sigma) susceptibility testing, 1% yeast extract medium was used.

Deletion of the *AGS* genes and construction of a triple *AGS* mutant. Genomic DNA was extracted as described by Girardin et al. (12). For Southern blot analysis, 10 μg of digested genomic DNA was size fractionated in 0.7% agarose and blotted onto a positively charged nylon membrane (Hybond-N+; GE Healthcare).

Deletion cassettes were constructed by joining both 5′- and 3′-flanking sequences of each gene to be deleted with the positively selectable marker *HPH*, *BLE*, or *PYRG*, using the overlap method and the primers described in Table S1 in the supplemental material (18). Upstream and downstream *AGS1*, *AGS2*, or *AGS3* sequences were amplified from AkuB^{ku80} genomic DNA. Hygromycin, phleomycin, and *PYRG* resistance cassettes were amplified from pAN 7.1, pAN 8.1, and pAB4-1, respectively (7, 20, 23). The *ags1*Δ::*HPH* (*ags1*Δ) single mutant was constructed by replacing *AGS1* with the *HPH* gene (conferring resistance to hygromycin). The *ags1*Δ::*HPH ags2*Δ::*BLE* (*ags1*Δ *ags2*Δ) double deletion mutant was constructed by replacing the *AGS2* open reading frame (ORF) with the *BLE* gene (conferring resistance to phleomycin) in the *ags1*Δ background. In the *ags1*Δ::*HPH ags2*Δ::*BLE ags3*Δ::*PYRG* (*ags1*Δ *ags2*Δ *ags3*Δ) triple deletion mutant, the *AGS3* ORF was replaced with the *PYRG* gene from *Aspergillus niger* (conferring the ability to grow on medium without uracil and uridine) (28) in the double deletion mutant (*ags1*Δ *ags2*Δ) background (see Fig. S1). Transformations were achieved by following an

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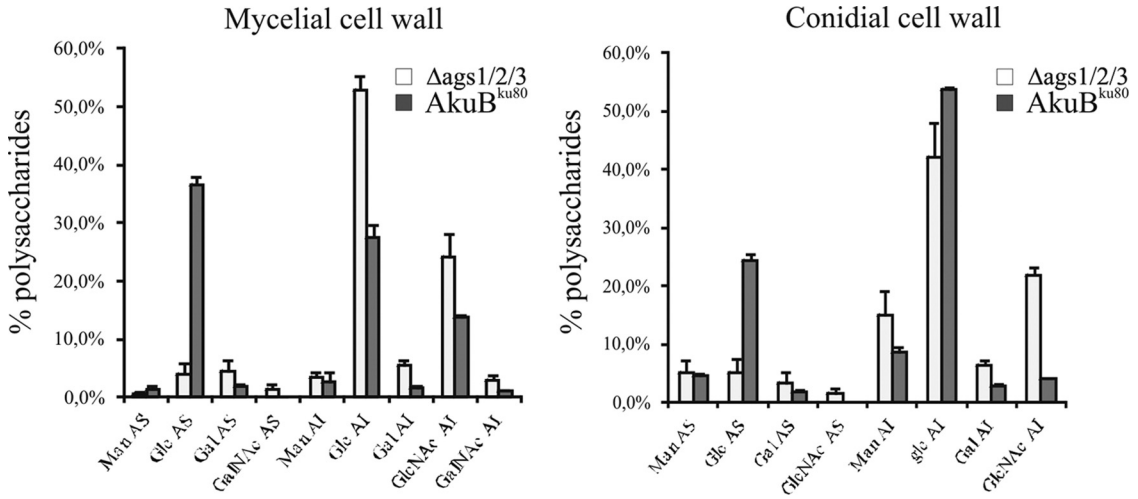

FIG 1 Cell wall compositions of the *ags1*Δ *ags2*Δ *ags3*Δ mutant and the AKuB^{ku80} parental strain. The monosaccharide compositions of the alkali-soluble (AS) and alkali-insoluble (AI) fractions are shown. Glc, glucose; Man, mannose; Gal, galactose; GlcNac, *N*-acetylglucosamine; GalNac, *N*-acetylgalactosamine.

electroporation or protoplast protocol (2, 19). The following day, drugs were added to the plates. The plates were incubated for 1 week at 20°C for the *ags1*Δ and *ags1*Δ *ags2*Δ strains and at 37°C for the *ags1*Δ *ags2*Δ *ags3*Δ strain.

For each transformation, transformants obtained under appropriate selective conditions were screened by PCR amplification using three pairs of primers (see Table S1 in the supplemental material). The first pair consisted of one primer designed to target outside the 5′ end of the deletion cassette coupled with one primer designed to target inside the resistance gene. The second pair of primers consisted of one primer designed to target outside the 3′ end of the deletion cassette coupled with one primer designed to target inside the resistance gene. The third pair of primers was designed to target inside the *AGS* gene. Positive amplicons obtained with only the first two pairs of primers indicated that the full-length deletion cassette was incorporated at the appropriate locus and that the *AGS* gene was deleted in these transformants. To confirm the results obtained by PCR, genomic DNA was digested by appropriate enzymes and analyzed by Southern blotting (see Fig. S1).

Complementation of the triple mutant. Strategies to complement the triple *AGS* mutant are available in the supplemental material.

Phenotypic analysis of mutants. The carbohydrate compositions of the cell walls of conidia and mycelia were determined as described previously, using three different batches of culture (21, 22). The alkali-insoluble (AI) and alkali-soluble (AS) fractions were extracted from the lyophilized cell wall (22). Monosaccharide composition was analyzed by gas chromatography after hydrolysis with 4 N trifluoroacetic acid for hexoses and 8 N HCl for hexosamines for 4 h at 100°C, followed by reduction and peracetylation of the alkali-insoluble and alkali-soluble fractions (22). α- and β1,3 glucans were determined by measuring the reducing sugar released by recombinant α- and β1,3 glucanases (2). An aggregation assay of germinating conidia was done as previously described (8). Nikkomycin Z susceptibility was tested following an adaptation of the resazurin method described by Clavaud et al. (5).

RESULTS AND DISCUSSION

Construction of a triple *AGS* mutant. A triple *AGS* mutant was generated through successive deletions of the three *AGS* genes (see Fig. S1 in the supplemental material). Deleting *AGS* genes in *A. fumigatus* has always been difficult. Originally, single mutants were obtained in a CBS144-89 wild-type background, with a transformation efficiency of <1% (2, 21). In spite of many attempts, no triple mutants were obtained using this parental strain. In order to improve the transformation efficiency, an AkuB^{ku80} derivative of the same strain, deficient for nonhomologous end joining and favoring homologous recombination, was used (6). Even though the transformation efficiency was improved, the total number of transformants per transformation remained very low (<6/transformation). Four *ags1*Δ mutants were obtained after 3 transformation experiments; 2 double mutant *ags1*Δ *ags2*Δ transformants were selected after 5 transformation experiments. It was verified that the phenotype of the *ags1*Δ mutant was the same in AkuB^{ku80} and CBS144-89. The *ags1*Δ *ags2*Δ mutant had the same phenotype (growth, sporulation, and cell wall composition) as the *ags1*Δ mutant (data not shown). Finally, after 3 transformations, a unique clone was isolated subsequent to the integration of the *ags3*Δ cassette at the *AGS3* position in the *ags1*Δ *ags2*Δ strain to create the triple *ags1*Δ *ags2*Δ *ags3*Δ mutant. The correct integration of the resistance marker at the right locus was verified by Southern blotting.

The triple *AGS* mutant of *A. fumigatus* is totally deficient in α1,3 glucans. The chemical composition of the *A. fumigatus* cell wall was investigated in the parental and triple *AGS* mutant strains. The ratios of AI concentration to AS concentration in the mycelial and conidial cell walls of the *ags1*Δ *ags2*Δ *ags3*Δ mutant were increased in comparison to those of the AkuB^{ku80} parental strain. Ratios of 10 and 2.6 were seen for the *ags1*Δ *ags2*Δ *ags3*Δ and parental strains, respectively, for the mycelium, with ratios of 8 and 2.2, respectively, for the conidia. The high alkali-insoluble/alkali-soluble hexose ratio was due to a reduction in the glucose content of the alkali-soluble fraction of the *ags1*Δ *ags2*Δ *ags3*Δ mutant: there was 4% glucose in the *ags1*Δ *ags2*Δ *ags3*Δ mycelium and 38% glucose in the AkuB^{ku80} mycelium, while there was 5% glucose in the *ags1*Δ *ags2*Δ *ags3*Δ conidia and 24% glucose in the AkuB^{ku80} conidia (Fig. 1). Unexpectedly, a small amount of glucan was found in the AS fraction of the *ags1*Δ *ags2*Δ *ags3*Δ mutant. However, after α1,3 glucanase treatment, the mycelial AS fraction of the parental strain also contained 4% glucan and the conidial AS fraction contained 7% glucan, similar to the amounts encountered in the triple *AGS* mutant. The remaining glucan in the α1,3 glucanase-treated mycelial and conidial AS fractions of the *ags1*Δ

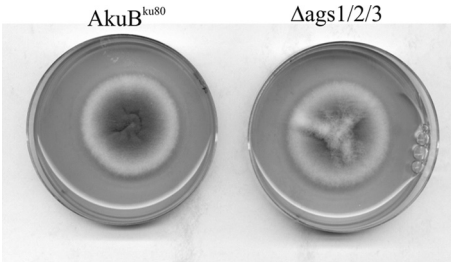

FIG 2 Colonies of the triple *AGS* mutant (*ags1*Δ *ags2*Δ *ags3*Δ) and the AkuB^{ku80} parental strain grown for 48 h at 37°C on Sabouraud agar medium.

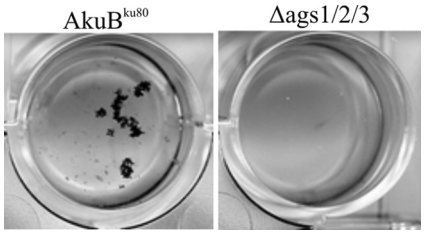

FIG 3 Conidial aggregation after 2 h of incubation at 37°C with shaking (150 rpm). Note the lack of conidial aggregation for the triple *ags1*Δ *ags2*Δ *ags3*Δ mutant.

*ags2*Δ *ags3*Δ mutant and parental strains was totally degraded by β1,3 glucanase (data not shown). This result showed that the remaining glucan was a β1,3 glucan. The origin and structure of this small amount of β1,3 glucan in the alkali-soluble fraction remain unknown. This result confirmed, however, that the triple *AGS* mutant was totally devoid of α1,3 glucans.

The decrease in the α1,3 glucan in the alkali-soluble fraction was compensated by an increase in the galactosamine present in the alkali-soluble and alkali-insoluble fractions (Fig. 1). Polygalactosamine constituted the galactosaminogalactan, which is also an amorphous polysaccharide of the *A. fumigatus* cell wall (9). β1,3 glucans and chitin were also increased in the mycelial alkali-insoluble fraction, whereas only chitin was increased in the conidial alkali-insoluble fraction (Fig. 1). Similarly, analysis of the cell wall of a *C. neoformans ags1*Δ mutant demonstrated that the loss of α1,3 glucans was accompanied by a compensatory increase in the chitin/chitosan concentration and a redistribution of β1,3 glucans between the cell fractions (26). The loss of α1,3 glucans in the cell wall of the *ags1*Δ *ags2*Δ *ags3*Δ mutant was compensated by an increase in the alkali-insoluble polysaccharide content (Fig. 1). The compensatory increase of the alkali-insoluble polysaccharide amount showed that *A. fumigatus* needed to reinforce the rigidity of the cell wall affected by the loss of α1,3 glucans. These compensatory reactions explain why the thicknesses of the cell walls of the triple mutant and parental strains observed by transmission electron microscopy were similar (data not shown). The increase in the amount of chitin in the cell wall of the *ags1*Δ *ags2*Δ *ags3*Δ mutant was correlated with a decrease in the susceptibility of the mutant to the chitin synthase inhibitor nikkomycin Z. MIC values for nikkomycin were 25 μg/ml and >200 μg/ml for AkuB^{ku80} and the *ags1*Δ *ags2*Δ *ags3*Δ mutant, respectively. Seven chitin synthases were found in the *A. fumigatus* genome (1).

α1,3 glucans are not essential in *A. fumigatus*. Unexpectedly, the growth of the triple mutant was similar to that of the AkuB^{ku80} strain in Sabouraud's, Brian's, complete, and minimal media at all temperatures and pHs tested (Fig. 2; data not shown). This result showed that α1,3 glucans were not essential in *A. fumigatus* and suggested that alkali-soluble polysaccharides of *A. fumigatus* did not play any structural role in the cell wall of this fungal species. Similarly, a strain of *H. capsulatum* that did not contain any α1,3 glucans in the cell wall grew like wild-type strains that contained more than 35% α1,3 glucans in the cell wall (16). In other fungi, such as *S. pombe* and *C. neoformans*, α1,3 glucans are essential for yeast morphogenesis (14, 26). Similar results were also obtained for other dimorphic pathogenic fungi in which the switch between β1,3 glucans and α1,3 glucans is associated with the changes between mycelium and yeast forms (15, 27). In *S. pombe*, the content of α1,3 glucans is 28%. Five *AGS* genes are present in *S. pombe*, but only *AGS1* of *S. pombe* is an essential gene (14). At a semipermissive temperature, the thermosensitive *ags1.1* mutant contains only 7% α1,3 glucans in its cell wall (14). The cells are rounded and pear-shaped, and the cell wall becomes looser and thicker than that of the parental strain.

The conidiogenesis of the triple mutant was slightly decreased, as found for the single *ags1*Δ and *ags2*Δ mutants, compared to that of the parental strain (2). The viability of the conidia was not affected, and the germination level of the triple *AGS* mutant was similar to that of the parental strain (data not shown). However, the analysis of the aggregation phenotype of the germinating conidia also confirmed that the triple *AGS* mutant did not contain α1,3 glucans in the cell wall. As reported earlier, conidia of the parental strain started to aggregate after 90 min of incubation in a culture medium at 37°C, as soon as α1,3 glucans emerged on the cell wall surface. After 3 h, large aggregates containing more than 95% of germinating conidia were seen for the parental AkuB^{ku80} strain (Fig. 3). Previous studies (8) have demonstrated that this aggregation depends exclusively on α1,3 glucan-α1,3 glucan interactions. In contrast, and in agreement with the lack of α1,3 glucans in the cell wall, no aggregation was observed with the germinating conidia of the *ags1*Δ *ags2*Δ *ags3*Δ mutant (Fig. 3).

Unfortunately, in spite of many transformation experiments using the various strategies summarized above, it was impossible to complement the triple *AGS* mutant. Reasons for these repeated failures remain unknown, but they could be due to the large size of the genes (>8 kb), their *in vivo* three-dimensional conformation, or the presence of one of these *AGS* genes (*AGS1*) in the subtelomeric region of a chromosome (chromosome 3), which is always difficult to manipulate genetically (11). Nevertheless, the lack of a growth phenotype in a triple *AGS* mutant totally devoid of α1,3 glucans showed definitively that the α1,3 glucans that are the major cell wall component are fully dispensable for *A. fumigatus* vegetative growth.

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