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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 brasilienisis, Histoplasma 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 ags1Δ 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 ags1Δ mutant, the mycelial cell walls of ags2Δ and ags3Δ 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 agsΔ mutants. Compensatory expression of the other members of the AGS family, which has been seen in all single agsΔ mutants, could explain the lack of a significant phenotype for each single agsΔ mutant (2, 21). For example, AGS3 and AGS2 showed increased expression in the ags1Δ mutant, and AGS1 expression compensated for the lack of AGS3 in the ags3Δ mutant (21).

Understanding the functional role of α1,3 glucans in A. fumigatus required the construction of a triple ags1Δ ags2Δ ags3Δ 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 ags1Δ ags2Δ ags3Δ 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 AkuBΔpyrGΔ strain was used as the parental strain (6). The AkuBΔpyrGΔ 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 (ags1Δ::HPH), with 5 μg/ml phleomycin (Invitrogen) added for the double deletion mutant strain (ags1Δ::HPH ags2Δ::BLE), and minimal medium was used for the selection of the triple deletion mutant strain (ags1Δ::HPH ags2Δ::BLE ags3Δ::PYRG). The triple deletion mutant strain (ags1Δ::HPH ags2Δ::BLE ags3Δ::PYRG; named the ags1Δ ags2Δ ags3Δ strain in this study) 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 Brain’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 AkuBΔpyrGΔ genomic DNA. Hygromycin, phleomycin, and PYRG resistance cassettes were amplified from pAN7.1, pAN8.1, and pAB4-1, respectively (7, 20, 23). The ags1Δ::HPH (ags1Δ) single mutant was constructed by replacing AGS1 with the HPH gene (confering resistance to hygromycin). The ags1Δ::HPH ags2Δ::BLE (ags1Δ ags2Δ) double deletion mutant was constructed by replacing the AGS2 open reading frame (ORF) with the BLE gene (confering resistance to phleomycin) in the ags1Δ background. In the ags1Δ::HPH ags2Δ::BLE ags3Δ::PYRG (ags1Δ ags2Δ ags3Δ) triple deletion mutant, the AGS3 ORF was replaced with the PYRG gene from Aspergillus niger (confering the ability to grow on medium without uracil and uridine) (28) in the double deletion mutant (ags1Δ ags2Δ) background (see Fig. S1). Transformations were achieved by following an

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RESULTS AND DISCUSSION

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 ags1Δ and ags1Δ ags2Δ strains and at 37°C for the ags1Δ ags2Δ ags3Δ 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 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\textsuperscript{kulu} 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/tranformation). Four ags1Δ mutants were obtained after 3 transformation experiments; 2 double mutant ags1Δ ags2Δ transformants were selected after 5 transformation experiments. It was verified that the phenotype of the ags1Δ mutant was the same in AkuB\textsuperscript{kulu} and CBS144-89. The ags1Δ ags2Δ mutant had the same phenotype (growth, sporulation, and cell wall composition) as the ags1Δ mutant (data not shown). Finally, after 3 transformations, a unique clone was isolated subsequent to the integration of the ags3Δ cassette at the AGS3 position in the ags1Δ ags2Δ strain to create the triple ags1Δ ags2Δ ags3Δ 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 α,1,3 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 ags1Δ ags2Δ ags3Δ mutant were increased in comparison to those of the AkuB\textsuperscript{kulu} parental strain. Ratios of 10 and 2.6 were seen for the ags1Δ ags2Δ ags3Δ 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 ags1Δ ags2Δ ags3Δ mutant: there was 4% glucose in the ags1Δ ags2Δ ags3Δ mycelium and 38% glucose in the AkuB\textsuperscript{kulu} mycelium, while there was 5% glucose in the ags1Δ ags2Δ ags3Δ conidia and 24% glucose in the AkuB\textsuperscript{kulu} conidia (Fig. 1). Unexpectedly, a small amount of glu- can was found in the AS fraction of the ags1Δ ags2Δ ags3Δ mutant. However, after α,1,3 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 α,1,3 glucanase-treated mycelial and conidial AS fractions of the ags1Δ

FIG 1 Cell wall compositions of the ags1Δ ags2Δ ags3Δ mutant and the AkuB\textsuperscript{kulu} 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-acetylgalactosamine; GalNac, N-acetylgalactosamine.
ags2Δ ags3Δ 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 α,1,3 glucans.

The decrease in the α,1,3 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 an amorphous polysaccharide of the H. capsulatum 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 ags1Δ mutant demonstrated that the loss of α,1,3 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 α,1,3 glucans in the cell wall of the ags1Δ ags2Δ ags3Δ 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 α,1,3 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 ags1Δ ags2Δ ags3Δ mutant was correlated with a decrease in the susceptibility of the mutant to the chitin synthesize inhibitor nikkomycin Z. MIC values for nikkomycin were 25 μg/ml and >200 μg/ml for AkuBku80 and the ags1Δ ags2Δ ags3Δ mutant, respectively. Seven chitin synthases were found in the A. fumigatus genome (1).

α,1,3 glucans are not essential in A. fumigatus. Unexpectedly, the growth of the triple mutant was similar to that of the AkuBku80 strain 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% α,1,3 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 ags1Δ and ags2Δ 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 α,1,3 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 α,1,3 glucans emerged on the cell wall surface. After 3 h, large aggregates containing more than 95% of germinating conidia were seen for the parental AkuBku80 strain (Fig. 3). Previous studies (8) have demonstrated that this aggregation depends exclusively on α,1,3 glucan-α,1,3 glucan interactions. In contrast, and in agreement with the lack of α,1,3 glucans in the cell wall, no aggregation was observed with the germinating conidia of the ags1Δ ags2Δ ags3Δ 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 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 α,1,3 glucans showed definitively that the α,1,3 glucans that are the major cell wall component are fully dispensable for A. fumigatus vegetative growth.

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