Identification of the UDP-glucose-4-epimerase required for galactofuranose biosynthesis and galactose metabolism in A. niger

Joohae Park, Boris Tefsen, Mark Arentshorst, Ellen Lagendijk, Cees AMJJ van den Hondel, Irma van Die and Arthur FJ Ram

Abstract

Background: Galactofuranose (Galf)-containing glycoconjugates are important to secure the integrity of the cell wall of filamentous fungi. Mutations that prevent the biosynthesis of Galf-containing molecules compromise cell wall integrity. In response to cell wall weakening, the cell wall integrity (CWI)-pathway is activated to reinforce the strength of the cell wall. Activation of CWI-pathway in Aspergillus niger is characterized by the specific induction of the agsA gene, which encodes a cell wall α-glucan synthase.

Results: In this study, we screened a collection of cell wall mutants with an induced expression of agsA for defects in Galf biosynthesis using a with anti-Galf antibody (L10). From this collection of mutants, we previously identified mutants in the UDP-galactopyranose mutase encoding gene (ugmA). Here, we have identified six additional UDP-galactopyranose mutase (ugmA) mutants and one mutant (named mutant #41) in an additional complementation group that displayed strongly reduced Galf-levels in the cell wall. By using a whole genome sequencing approach, 21 SNPs in coding regions were identified between mutant #41 and its parental strain which changed the amino acid sequence of the encoded proteins. One of these mutations was in gene An14g03820, which codes for a putative UDP-glucose-4-epimerase (UgeA). The A to G mutation in this gene causes an amino acid change of Asn to Asp at position 191 in the UgeA protein. Targeted deletion of ugeA resulted in an even more severe reduction of Galf in N-linked glucans, indicating that the UgeA protein in mutant #41 is partially active. The ugeA gene is also required for growth on galactose despite the presence of two UgeA homologs in the A. niger genome.

Conclusion: By using a classical mutant screen and whole genome sequencing of a new Galf-deficient mutant, the UDP-glucose-4-epimerase gene (ugeA) has been identified. UgeA is required for the biosynthesis of Galf as well as for galactose metabolism in Aspergillus niger.

Keywords: Filamentous fungi, Cell wall, Cell wall integrity, Galactofuranose, Illumina sequencing, Whole genome sequencing, SNP analysis, UDP-glucose-4-epimerase, Mutant screen, Galactose

Background

The cell wall is an essential component of the fungal cell. Cells can survive the enzymatic removal of the cell wall but the resulting protoplasts need to be stabilized in an environment with high osmolarity to withstand internal turgor pressure. The cell wall of filamentous fungi consists mainly (90%) of polysaccharide material, including polymers of glucose (β-1,3- and β-1,6-glucans, α-1,3-glucan), N-acetylglucosamine (chitin), mannose and galactofuranose (galactomannan), galactoaminogalactan and of about 10% of cell wall glycoproteins (galactomanno-proteins) [1-3]. The different glycoconjugates are either synthesized at the plasma membrane by specific cell membrane-localized synthases (e.g. chitin, β-1,3- and α-1,3-glucan) or preassembled in the secretory pathway (galactomannan and galactomannoproteins). During or after transport over the membrane, polymers are cross-linked
with each other via covalent or hydrogen bonds to create a sturdy cell wall.

We and others have previously shown that galactofuranose (Gal) is an important component of the cell wall in *Aspergillus* species. It is found in several glycoconjugates including galactomannan, secreted and cell wall proteins via N- and O-linked chains, and glycosphingolipids (see for review [4]). A key enzyme in Gal biosynthesis is UDP-Gal transferase (UgmA), which converts the pyranose form of UDP-galactose (UDP-Galp) into UDP-Gal. Only UDP-Galp can be transported into the Golgi where Galp is used as a donor sugar for the synthesis of Gal-containing structures [5-7]. Gene disruption approaches of the *ugmA* gene in *Aspergillus niger*, *A. fumigatus*, *A. nidulans* have shown that Gal biosynthesis is required for hyphal morphogenesis and cell wall architecture [8-10], whereas disruption of the *ugmA* homolog in *Cryptococcus neoformans* did not have an apparent growth phenotype nor did it affect capsule formation [11]. Inactivation of the *ugmA* genes in Aspergillus results in an increased sensitivity towards cell wall assembly interfering drugs such as calcofluor white (CFW), indicating that galactofuranose-containing glycoconjugates are necessary for maintaining the integrity of the cell wall [8].

In *A. niger* the *ugmA* gene was identified in a screen for mutants in which the cell wall stress reporter gene *agsA* was constitutively induced [8]. Besides UgmA, proteins required for the biosynthesis of Gal-containing glycoconjugates have been identified in both *A. nidulans* and *A. fumigatus*. These proteins include UgeA/Uge5, encoding the UDP-glucose-4-epimerase necessary for the synthesis of UDP-Galp [12,13], UgtA/Glb encoding a Golgi localized UDP-Galp transporter protein [5,6] and GfsA encoding a Galp transferase [7].

In this study, we have screened a collection of 240 cell wall mutants with induced *agsA* expression [8] for mutants that do not secrete Galp-containing glycoconjugates into the growth medium. In addition to a large complementation group of 9 *ugmA* mutants, one additional Galp-low mutant (#41), belonging to a different complementation group, was identified. Whole genome sequencing of this mutant revealed that the newly identified Galp-deficient mutant contains a mutation in gene *An14g03820*. This gene is predicted to encode a putative UDP-glucose-4-epimerase gene (*ugeA*), required for the biosynthesis of Galp as well as for Galp metabolism in *Aspergillus niger*.

**Results**

**Screening of Galp-deficient mutants within 240 *A. niger* cell wall mutants**

To identify additional genes involved in the biosynthesis of cell wall galactomannan in *A. niger*, we screened a collection of 240 cell wall mutants for Galp-deficient strains. Galp containing structures such as galactomannans and N- or O-glycosylated proteins are secreted in the medium and therefore analysis of the medium for the presence of Galp using an antibody can identify Galp minus mutants. From the collection of cell wall mutants, we previously identified the *A. niger* UDP-galactopyranose mutase (UgmA) as an essential protein for the formation of Galp-containing cell wall glycoconjugates ([8], see below). The selection of the three *ugmA* mutants in that study was not based on their Galp phenotype, but on their Calcofluor white- and SDS-hypersensitive phenotype. Here, all 240 mutants were grown in liquid medium and 2 μl of medium was used in a dot blot analysis using the anti-Galp antibody L10 [14] as described in Materials and Methods (Figure 1). The screening confirmed the absence of Galp in the three *ugmA* mutants already identified (6.13#44, 15.4#17, and 6.13#50) [8], and identified six additional Galp-minus mutants (15.4#30, 15.4#18, 15.4#5, 15.4#50, 15.4#57 and 6.47#41). A heterokaryon complementation test using the Δ*ugmA* strain was performed to determine whether the newly identified mutants were also mutated in *ugmA*. In this test, spores of mutants of interest are inoculated towards cell wall assembly interfering drugs such as calcofluor white (CFW), indicating that galactofuranose-containing glycoconjugates are necessary for maintaining the integrity of the cell wall [8].

Mutant #41 has reduced levels of Galp

To address the Galp-deficient phenotype of the #41 mutant further, medium samples of mutant #41 were analysed using the Platelia assay. This quantitative assay for Galp detection uses the monoclonal EB-A2 antibody which recognizes Galp-moieties on galactomannoproteins [15]. As shown in Figure 3A, titration of the medium samples revealed an approximately 10-fold reduced amount of Galp-reactive epitopes produced by the #41 mutant compared to that of its parental strain RD647.
As controls, high reactivity with medium from wild type N402 and no reactivity with medium from the ΔugmA strain were measured in this assay. Western blot analysis of extracellular medium proteins using the L10 antibody also detected less Gal\(\beta\)epitopes on secreted glycoproteins in contrast to the wild type strains N402 and RD6.47, but clearly more than in the medium of the ΔugmA strain (Figure 3B). Detection of \(N\)-glycans with the lectin ConcanavalinA (ConA) was performed as a control.

To obtain more insight into the presence of Gal\(f\) in the cell wall of the #41 mutant, galactomannan from two Gal\(f\) mutants (ΔugmA and #41), and the wild-type strain (N402) was isolated essentially as described previously by Bardalaye and Nordin, 1977 [16] (see Methods for details). A titration of the purified galactomannan fraction was applied to the Platelia assay, revealing again a lowered reactivity of the Gal\(f\)-antibody with the galactomannan derived from #41 compared to the wild-type strain (Figure 3C),
whereas the polysaccharide isolated from ΔugmA was not reactive at all. This indicated that there are less Gal\(f\)-moi-eties present on the galactomannan from #41 compared to wild type galactomannan. To confirm this result, the monosaccharide composition of the galactomannan fraction of these strains was analysed by HPAEC analysis (DIONEX system) after hydrolysis (Figure 3D). As expected, no Gal\(f\) was detected in the hydrolyzed polysaccharide isolated from the ΔugmA mutant. The ratio Gal\(f\)/Man of the wild-type strains was found to be well above 2 (2.2-2.8), while this ratio was only 0.5 for the #41 mutant. These results demonstrate that the amount of Gal\(f\) is reduced more than 4-fold in the #41 mutant compared to its parent strain. This lowered amount of Gal\(f\) apparently has escaped detection by the L10 antibody in our initial screening by dot blot, leading to its discovery (Figure 1), but clearly is detected by the Platelia assay (Figure 3A and 3C), using the EB-A2 antibody, as well as by Western blot analysis using the L10 antibody (Figure 3B).

**Genomic characterization of mutant #41 by whole genome sequencing**

An *A. niger* genomic cosmid library [17] was used in several unsuccessful attempts to complement the reduced sporulating growth phenotype of mutant #41. As an alternative approach, the genome of both the parental strain (RD6.47) and the #41 mutant were sequenced by pair-end Illumina sequencing. A single nucleotide polymorphism (SNP) analysis between the two strains was performed and 78 SNPs were identified (Additional file 1: Table S1). These SNPs were analysed further by determining whether a particular SNP was located in a predicted ORF and whether this SNP affected the amino acid sequence. We identified 21 SNPs in genes in the #41 mutant that caused changes in the amino acid sequence (Additional file 2: Table S2). Most of these SNPs seemed unrelated to Gal\(f\)-biosynthesis, but the mutation in gene An14g03280 encoding a putative UDP-glucose-4-epimerase seemed important as, it was recently published that the UDP-glucose-4-epimerase (UgeA) is needed for Gal\(f\) biosynthesis in *A. nidulans* [13]. The mutation in the *ugeA* (An14g03280) gene of mutant #41 (A to G) caused the change of a codon from AAC to GAC which consequently resulted in the change of Asn to Asp at position 191 in the UgeA protein. The mutation identified by the Illumina genome sequencing approach was confirmed by PCR amplification of the *ugeA* locus of the #41 strain followed by direct sequencing.
Disruption of the A. niger ugeA gene and phenotypic analysis

To confirm that the mutation in An14g03820 (ugeA) caused the GalF-low phenotype in mutant #41, a pyrG based gene deletion cassette was made. The deletion cassette (pΔugeA::pyrG) was transformed to MA169.4 and uridine prototrophic transformants were purified. During purification of the primary transformants it was found that most of the transformants displayed a reduced growth and a reduced sporulation phenotype, which resembled the phenotype of the #41 mutant. Subsequent diagnostic PCR and Southern blot analysis proved that the transformants with the growth phenotype contained an ugeA deletion (data not shown). Further phenotypic characterization of the ΔugeA mutant showed similar morphological alterations (increased branching and irregular length of hyphal compartments) as found for the #41 mutant (data not shown).

As demonstrated above, very low levels of GalF were observed in the dot blot (Figure 1) and Platelia assay (Figure 3A) for the #41 mutant. Interestingly, the amount of GalF in the ΔugeA strain was strongly reduced compared to the #41 mutant, indicated by the complete lack of signal on the dot blot (Figure 4) and a further 10-fold reduction of the GalF-signal in the Platelia assay (Figure 3A). These results suggest that the UgeA protein in #41 is still slightly active and able to convert some UDP-Glc to UDP-Gal, which is subsequently utilized for GalF-biosynthesis. Despite the observation that more GalF is present in the #41 mutant compared to the ΔugeA mutant, both mutants display similar phenotypes, although some subtle differences could be seen between the #41 and the ΔugeA mutants. The #41 mutant seems to sporulate slightly more intense on the MM plate and grows somewhat better on the 0.005% SDS plate (Figure 5).

To further confirm that the mutation in ORF An14g03820 (ugeA) in the #41 mutant and the deletion of ugeA are the cause of the observed phenotypes, a complementation analysis was performed. Therefore, pyrG derivatives were obtained from the #41 and ΔugeA mutants by selecting on 5’FOA plates. The ugeA gene was PCR amplified and cloned into an autonomously replicating vector (pAMA-pyrG, [18]) and used for complementation. Transformation of the pAMA-ugeA plasmid to the #41 and ΔugeA mutants
complemented the reduced growth and sporulation phenotype (Figure 6A and B) as well as the Gal\(f\)-negative phenotype in the dot blot analysis (data not shown). Control transformations with the empty plasmid (pAMA-pyrG) or the pAMA-ugmA-pyrG gene were also performed. In general, the transformation of these control plasmids did not complement the sporulation defect of the \#41 and \(\Delta\)ugeA mutants. Unexpectedly, we occasionally (with a frequency of 0.2%-1%) obtained transformants with the empty or \(\text{ugmA}\) plasmid that sporulated well and grew like the complemented strain. From the original transformation plates of the \(\Delta\)ugeA mutant with the pAMA-pyrG or the pAMA-ugmA-pyrG plasmids, two transformants were purified with the improved sporulation phenotype. The pAMA-pyrG or pAMA-ugmA-pyrG transformant that sporulated well were also Gal\(f\)-positive in the dot blot analysis (data not shown). The reversion of the phenotype might be caused by second site suppressor mutations or by increased expression of redundant genes (see Discussion).

### UgeA is required for growth on galactose in \(A.\) niger

Based on sequence comparisons, UgeA (An14g03820) is predicted to encode a UDP glucose-4-epimerase and the result above clearly indicate that this gene is most likely responsible for the synthesis of UDP-Gal\(p\), which is further converted to UDP-Gal\(f\) by the UgmA enzyme. \(A.\) niger contains in addition to UgeA (An14g03820) two other homologous genes predicted to encode UDP glucose-4-epimerases (An12g10410 and An02g11320). The presence of three putative UDP glucose-4-epimerases in \(A.\) niger is similar to \(A.\) fumigatus in which the function of the three genes (uge3, uge5 and uge4) was recently analysed [12]. Based on homology, the \(A.\) niger UgeA is most similar to...
the *A. fumigatus* Uge5 protein and the UgeA protein of *A. nidulans* (See Table 1 for amino acid identities of the Uge homologs between the three species). To test whether the *A. niger ugeA* gene is required for growth on galactose, mutant #41, ΔugeA and their respective parental wild type strains were inoculated on plates containing 50 mM galactose and 0.3 mM arabinose. The supplementation of a second carbon source in addition to galactose is included because *A. niger* spores are unable to germinate on galactose alone [19-21]. As shown in Figure 6C, the #41 and ΔugeA mutants do not grow on galactose medium indicating that ugeA is required for growth on galactose. The inability to grow on galactose of the ugeA strain is not a consequence of its inability to produce Galβ as the ugmA deletion strain grows normally on galactose (Figure 6C).

**Discussion**

Screening of a collection of *A. niger* cell wall mutants, which were previously isolated by induced expression of the cell wall stress reporter gene (*agsA*), identified in

**Table 1 Comparison of the percentage of amino acid identity between Uge proteins of *A. niger, A. fumigatus*, and *A. nidulans***

| Species     | Protein ID | A. niger | A. fumigatus | A. nidulans |
|-------------|------------|----------|--------------|-------------|
|             |            | UgeA     | UgeB         | UgeC        | Uge3        | Uge4        | Uge5        | UgeA     | UgeB       |
| *A. niger*  | An14g03820 | 100*     | 40           | 39          | 39          | 37          | 94          | 94        | 39         |
|             | An12g10410 | 100      | 66           | 58          | 59          | 41          | 39          | 58        | 81         |
|             | An02g11320 | 100      | 66           | 86          | 59          | 40          | 40          | 81        |            |
| *A. fumigatus* |         |          | 100          | 58          | 39          | 39          | 84          |           |            |
|             | An14g03820 | 100      | 66           | 86          | 59          | 40          | 40          | 81        |            |
| *A. nidulans* |        |          | 100          | 58          | 39          | 39          | 84          |           |            |

* values indicate percentage of amino acids identities after pairwise alignment using BLASTP at http://blast.ncbi.nlm.nih.gov/Blast.cgi.
total 9 mutants which did not secrete detectable levels of Galf-containing glycoconjugates into the medium. Three mutants were identified previously [8] and six new Galf-deficient mutants were additionally found in this screen. Interestingly, the mutants fell into two unevenly distributed complementation groups. A large group (represented by eight alleles) consisted of mutants in the ugma gene and the other complementation group only contained a single representative with a mutation in the ugeA gene. The reason for this uneven distribution among the two complementation groups is not clear as both the ugma and the ugeA mutants have very similar phenotypes when inactivated (Figures 4 and 5). Besides UgeA and UgmA, which are required for the synthesis of the UDP-Galf, proteins involved in subsequent steps of the synthesis of Galf-containing glycoconjugates have been recently identified and include a specific UDP-Galf transporter (GlfB in A. fumigatus [5] and UgtA in A. nidulans [6] and a Galf-transferase [7]. Mutants in the homologs of those genes in A. niger were not identified in our cell wall mutant collection. Analysis of the A. niger genome showed the presence of two homologs of GlfB/UgtA and three homologs of the GfA protein (data not shown). The inability to detect representative mutants in the cell wall mutant collection suggests possible redundant functions of the pairs of genes in A. niger. Indeed, the two A. niger Galf transporters only display a Galf minus phenotype when both genes were deleted (Park and Ram, unpublished data).

This study represents the first example of the power of genome sequencing for characterizing classically obtained mutants in A. niger. The mutant #41 strain was created by UV mutagenesis which is known to preferably induce mutations (purine to purine (A-G) or pyrimidine to pyrimidine (T-C) mutations. From the 78 SNPs a majority of 49 (63%) SNPs were transitions and 29 (27%) were found to be transversions (purine to pyrimidine or reverse) (Additional file 1: Table S1). Of the 29 transversions, a high number of A to T transitions were found. The total number of mutations (21) in non-exonic regions was relative low taking into account the relative stringent dosis of UV treatment (66% survival). This number of SNPs relative low taking into account the relative stringent dosis of UV treatment (66% survival). This number of SNPs included mutations in 21 different genes. The high frequency of suppression of the ΔugeA phenotype after transformation. Although the phenotype of the #41 and ΔugeA mutants were stable during normal propagation, transformation of both strains yielded regularly suppressor mutations that were restored in their ability to produce Galf-decorated galactomannoproteins and were also restored in their ability to grow on galactose (data not shown). A possible mechanism to explain the development of suppressors include activation of expression of one of the uge genes, or occurrence of a mutation in one of these genes that changes substrate specificity.

**Conclusion**

By screening a collection of UV-generated cell wall mutants, a new Galf-deficient mutant was identified. We used whole genome sequencing to identify the mutation responsible for this mutant phenotype. Complementation and targeted deletion studies confirmed that the UDP-glucose-4-epimerase gene (ugeA) is required for
the biosynthesis of Gaf as well as for galactose metabolism in *Aspergillus niger*.

**Methods**

**Strains and culture conditions**

The *Aspergillus niger* strains used in this study are listed in Table 2. Strains were grown on minimal medium (MM) [23] containing 1% (w/v-1) glucose as carbon source or complete medium (CM) containing 0.5% (w/v-1) yeast extract, 0.1% (w/v-1) casamino acids in addition to MM. When required, plates were supplemented with 10 mM uridine. 5′ FOA selection for the selection of pyrG− strains was performed as described previously [24]. For the plate growth assays, strains were grown on MM plates containing 25 mM glucose, 25 mM galactose, or 25 mM galactose and 3 mM arabinose. For the heterokaryon assay, MM-plates were supplemented with 0.005% SDS.

**Screening methods to identify Gaf minus mutants**

Strains from the collection of 240 cell wall mutants of *A. niger* [8] were grown in 25 ml CM in 50 ml tube Greiner tube for 24 h at 30°C. Medium samples were filtered over a Whatman glass microfiber filter and 2 μl medium was spotted on nitrocellulose blotting paper. Blots were incubated with the L10 monoclonal anti-GalF-antibody (1:10) in TSMT (TSM [20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM CaCl2, 2 mM MgCl2] with 0.05% Tween-20) supplemented with 5% BSA or with peroxidase labeled ConA (ConA-PO EY Laboratories, USA) used in a 1:500 dilution in PBS containing 0.05% (v/v).

| Strain     | Description                                      | References          |
|------------|--------------------------------------------------|---------------------|
| N402       | cspA1 derivative of ATCC9029                      | Bos et al. 1988     |
| MA70.15    | ΔkusA::amdS, pyrG−                                 | [25]                |
| MA169.4    | ΔkusA:DR_amdS:DR, pyrG−                          | [18]                |
| MA234.1    | MA169.4 transformed with pAB4.1 to make strain pyrG+ | Arentshorst, unpublished |
| RD6.47     | galF− mutant derived from RD6.47                  | [8]                 |
| RD6.47#41  | pyrG− derivative of RD6.47#41                    | This study          |
| RD6.47#41/pAMA_ugeA |                                    | This study          |
| RD6.47#41/pAMA_ugmA |                                     | This study          |
| RD6.47#41/pAMA    |                                     | This study          |
| RD6.47#41/pAMA_ugeA |                                    | This study          |
| JH12.1     | pyrG- derivative of JH12.1                       | This study          |
| JH21.1     | ΔugeA/pAMA_ugeA                                  | This study          |
| JH21.1.1   | ΔugeA/pAMA_ugmA                                  | This study          |
| JH21.1.3   | ΔugeA/pAMA                                      | This study          |
| MA87.6     | ugmA (An14g03820):pyrG in MA70.15                 | [8]                 |
| MA247.2.1b | pyrG− derivative of MA87.6                       | This study          |
| MA247.2.1b.1|                                    | This study          |
| MA247.2.1b.2|                                    | This study          |
| MA247.2.1b.3|                                    | This study          |
| RD15.4     | pPagsA-H2B-GFP-TrpC-pyrG+ and pPagsA-amdS-Tamds/pAN7.1 | [8]                 |
| RD15.4#5   | galF− mutant derived from RD15.4                  | [8]                 |
| RD15.4#18  | galF− mutant derived from RD15.4                  | [8]                 |
| RD15.4#30  | galF− mutant derived from RD15.4                  | [8]                 |
| RD15.4#50  | galF− mutant derived from RD15.4                  | [8]                 |
| RD15.4#57  | galF− mutant derived from RD15.4                  | [8]                 |
| RD6.13#44  | ugmA mutant derived from RD6.13                   | [8]                 |
| RD6.13#47  | miaA mutant derived from RD6.13                   | [8]                 |
| RD6.13#50  | miaC mutant derived from RD6.13                   | [8]                 |

\[\text{pyrG}^+=\text{pyrG plus (uridine prototroph); pyrG}^-=\text{pyrG minus (uridine auxotroph); pyrG}^{*}\text{= mutant pyrG allele for targeting at the pyrG locus.}\]
Isolation of cell wall galactomannan

Cell walls were isolated from *A. niger* wild type strain N402 and the #41 mutant strain after growth in CM for 24 h at 30°C at 250 rpm. Spores (1 × 10⁹) were inoculated in 1 L of CM in a 2 L Erlemeyer. The mycelium of both strains was isolated by filtering over myrcloths and grinded in liquid nitrogen using a pestle and mortar and the broken mycelia washed 3 times with 1 M NaCl and 3 times with MilliQ at 4°C by centrifugation (3600 rpm, 10 min). Successful breakage of the mycelia was confirmed by microscopy. Isolated cell walls were lyophilized for 24 h using 1.0 gram of cell walls (dry weight) for both the N402 and the #41 strain. The galactomannan fraction was isolated according to Bardalaye and Nordin [16]. The yield of purified galactomannan was 21.2 mg and 10.4 mg from 1 gram of freeze dried cell walls for the N402 and #41 strain, respectively.

Platelet assay

Microtiter plate wells containing coated antibody EB-A2, which recognizes Galf-moieties on galactomannan [14] were filled with 50 μl EB-A2 conjugated to HRP (all from Platelia Aspergillus EIA kit, Bio-Rad). Supernatant of *A. niger* strains or purified galactomannan was added to the wells and incubated for 90 min at 37°C. After washing 5 times, 200 μl 3,3’,5,5’-tetramethylbenzidine (TMB) detection mixture was added and samples were incubated for 30 min at ambient temperature in the dark. After stopping the coloring reaction by addition of 100 μl 1.5 N H₂SO₄, relative amounts of Galf on galactomannan were determined by measuring the optical density (OD) by spectrophotometry at 450 nm.

Monosaccharide analysis by HPAEC

Galactomannan isolated from *A. niger* was analyzed for its monosaccharide composition by High-Performance Anion Exchange Chromatography (HPAEC), using the method described by [26] with minor modifications. In summary, chromatography of the samples was performed in a Dionex, Bio-LC system, using a CarboPac PA 10 column (250 x 2 mm) in combination with a CarboPac guard column (2x50mm), Dionex Corp. Around 2 mg galactomannan was dissolved in 2 ml water and incubated with 360 μl of TFA for 5 h at 100°C. Hereafter, 3 ml MilliQ water was added and samples were freeze dried overnight. The pellets were dissolved in 100 μl water. After diluting 1:100, 20 μl (equivalent to 4 μg polysaccharide) was injected onto the column. Monosaccharides were eluted isocratically using H₂O and 1 M NaOH in a ratio of 80:20 for 30 min. The column was washed with water for 10 min and equilibrated with 250 mM NaOH for 10 min before every injection.

Western blot analysis

Medium samples of the various strains were obtained as described for the dot-blot analysis and 20 μl of culture filtrate was used for Western blot analysis. SDS-PAGE and blotting were carried out as described [27]. For labeling with the anti-Galf antibody (L10), the membrane was blocked with 5% low-fat milk in TTBS (Tris-buffered saline, 0.05% Tween-20), and Galf was detected using the L10 antibody (1/5,000) overnight, followed by a goat anti-rabbit horseradish peroxidase secondary antibody (1/20,000) for 1 h. Detection was performed using a chemiluminescence kit (Thermo Scientific Pierce), according to manufacturer’s instructions.

Genetic methods

Fungal transformations were done according the protoplast method described by [24]. Complementation tests were done using the heterokaryon test. 2 μl of spores (1 × 10⁶ spores/ml) of strains to be tested were inoculated on MM plates containing 0.005% SDS approximately 1 cm apart from each other. Plates were incubated for 3 days at 30°C and analyzed for a zone of conidiation of heterokaryotic mycelium.

Plasmid construction

For the construction of the *ugeA* deletion cassette the Multisite GatewayR Three-Fragment Vector Construction kit was used. As a marker for deleting the *ugeA* gene, the *pyrG* marker of *A. oryzae* was used. To facilitate removal of the *AopyrG* marker, *A. nidulans* tTrpC repeats were cloned around the *pyrG* gene [28]. The TrpC-pyrG-TrpC fragment was PCR amplified using primer listed in Table 3 and cloned in pDonor221. The 5’ and 3’ flanking regions of *ugeA* were PCR amplified using primers with appropriate attB sites (Table 3) and the 799 bp fragments were cloned into pDONR P4-P1R and pDONR P2R-P3 respectively. The subsequent LR reaction was performed using pDONR_ugeA5, pDONR_ugeA3 and pDONR_TrpC_pyrG_TrpC and pDEST R4-R3 Vector 2 to create the *ugeA* deletion plasmid. The final construct was verified by restriction analysis and sequencing.

For the complementation analysis the autonomously replicating vector pMA172 was used [18]. The *ugeA* or *ugmA* genes including promoter and terminator regions were PCR amplified using primers listed in Table 3, cloned into pJet2.1 and verified by sequencing. The *ugeA* and *ugmA* genes were reisolated as *Sma*I (*ugeA*) or *Not*I (*ugmA*) fragments and cloned into the unique *Sma*I or...
Table 3 The primers used in this study

| Name                      | Sequence 5′-3′                                                     | Application                                      |
|---------------------------|-------------------------------------------------------------------|--------------------------------------------------|
| attB1_TrpC_CFP_F           | ggggacaacacctttcactattaataaagttgGGATGGACAGCTGTGACAGTTAAGTAA      | Amplification TrpC-cfp-GFP-TrpC cassette         |
| attB2r_TrpC_R             | ggggacaacacctttcactattaataaagttgGGATGGACAGCTGTGACAGTTAAGTAA      | Amplification TrpC-cfp-GFP-TrpC cassette         |
| attB4_ugeAF               | ggggacaacacctttcactattaataaagttgGGATGGACAGCTGTGACAGTTAAGTAA      | Amplification S′ flank  ugeA disruption cassette |
| attB1r_ugeAR              | ggggacaacacctttcactattaataaagttgGGATGGACAGCTGTGACAGTTAAGTAA      | Amplification S′ flank  ugeA disruption cassette |
| attB2r_ugeAF              | ggggacaacacctttcactattaataaagttgGGATGGACAGCTGTGACAGTTAAGTAA      | Amplification S′ flank  ugeA disruption cassette |
| attB3r_ugeAR              | ggggacaacacctttcactattaataaagttgGGATGGACAGCTGTGACAGTTAAGTAA      | Amplification S′ flank  ugeA disruption cassette |
| ugmAP2r-NotI             | aaggaaaaaaaacggccggtccggtTGGACTCCATAGGCCCTGGTAGAA                | Amplification ugmA for complementation and sequencing |
| ugmAP2r-NotI             | aaggaaaaaaaacggccggtccggtTGGACTCCATAGGCCCTGGTAGAA                | Amplification ugmA for complementation and sequencing |
| ugeAfw-Smal               | ctcgggtcgggtTGGCAAGGAAGAGGTGAAG                                    | Amplification ugeA for complementation and sequencing |
| ugeArev-Smal              | ctcgggtcgggtTGGCAAGGAAGAGGTGAAG                                    | Amplification ugeA for complementation and sequencing |

Italic letters indicate attB sites, restriction sites are shown in bold.

NotI site of pMA172 to give pAMA-ugeA and pAMA-ugmA respectively.

Genome sequencing and SNP identification

Genomic DNA isolations of strains 6.47 (parental strain) and #41 (mutant strain) were performed as described [24]. Genomic DNA was further purified using Macherey-Nagel NucleoBond Xtra columns. The Illumina Paired-End sequencing was performed by ServiceXS using Illumina kits (cat# 1001809 and 1005063) and protocols according to the instructions by the supplier. The quality and yield after sample preparation were checked and were consistent with the expected size of 300 bp after excision from gel. Clustering and DNA sequencing using Illumina cBot and HiSeq 2000 was performed according to manufacturer’s protocols. A total of 6.5 pmol of DNA was used. Two sequencing reads of 100 cycles each using Read1 sequencing and Read2 sequencing primers were performed with the flow cell. For strains 6.47 and #41, 3.1 and 3.3 Gb of DNA sequence was obtained respectively. The genome sequences are available on request. Image analysis, base calling and quality check was performed with the Illumina data analysis pipeline RTA v1.13.48 and/or OLB v1.9/CASAVA v1.8.2. SNPs between the two strains (mutant #41 and parental strain 6.47), were identified using A. niger strain ATCC1015 (http://genome.jgi-psf.org/pages/search-for-genes.jsp?organism=A.\ niger) as a reference genome. Low quality bases were removed from the raw sequencing data. A Q25 phred score was used as a minimum and bases with phred scores below were removed and reads containing these bases were split. If the resulting reads from splits were shorter than 40 bases they were removed altogether. Alignment of filtered reads was performed with BWA (version 0.5.9) which lays the foundation for finding SNPs. For each SNP it was verified whether the SNP was in a predicted protein encoding region using the A. niger 3.0 genome at JGI using the SNP coordinates.

Availability of supporting data

The data set(s) supporting the results of this article are included within the article and its additional files. Genome sequences of RD6.47 and mutant #41 are available upon request.

Additional files

Additional file 1: Tabel S1. List of all SNPs identified in mutant #41.
Additional file 2: Tabel S2. SNPs in coding regions in #41.
Additional file 3: Figure S1. The Asn to Asp mutation at position 191 in mutant #41 probably leads to misorientation of the substrate-orientation YFN-domain in UgeA. A) Protein alignment of the UgeA homologues from A. niger, A. nidulans, human and E. coli. Residues interacting with carbohydrate substrate are indicated with a black star, residues interacting with NAD substrate are indicated with a yellow star, the mutation identified in mutant #41 (N191D) is indicated with the blue arrow. B) Cartoon representation of the crystal structure from A. nidulans (PDB ID: 4LIS, [22], with β − strands 6 and 7 in red, residues interacting with carbohydrate substrate in black C) Space-filling model of UgeA; showing that the key enzymatic residues are located on the inside of UgeA D) Space-filling model of selected amino acid residues of UgeA: N191 in blue, residues forming β − strands 6 and 7 in red, residues interacting with carbohydrate substrate in black.

Competing interest

The authors declare that they have no competing interests.

Authors’ contribution

JP, BT and EL carried out the immunological analyses, JP and MA carried out the molecular genetic studies, JP, BT, ID, CH and AR designed the initial experiments. All authors contributed to data interpretation and writing. All authors read and approved the final manuscript.

Acknowledgements

We thank Denise van Haren and Esther Goedkoop for their help in carrying out experiments on the complementation and genome analysis and Peter Eijsser for constructing the ugeA deletion strain. We thank Frank Ebel for the L10 antibody and Annika Pettersson and Dorien van ’t Oever for the Platelia deletion strain. We thank Frank Ebel for the L10 antibody and Annrika Pettersson and Dorien van ’t Oever for the Platelia.

Author details

1Institute of Biology Leiden, Molecular Microbiology and Biotechnology, Leiden University, Sylviusweg 72, 2333, BE, Leiden, The Netherlands.
2Department of Molecular Cell Biology and Immunology, VU University
Medical Center, van den Boechorststraat 7, 1081, BT, Amsterdam, The Netherlands. Current address: Department of Biological Sciences, Xi’an Jiaotong Liverpool University, 111 Ren Ai Road, Dushu Lake Higher Education Town, Suzhou Industrial Park, Suzhou, Jiangsu 215123, China.

Received: 21 August 2014 Accepted: 1 September 2014 Published online: 14 October 2014

References

1. Bernard M, Latgé JP. Aspergillus fumigatus cell wall: composition and biosynthesis. Med Mycol 2001, 39(Suppl 1):9–17.
2. Gustafsson A, Clevea C, Amananda V, Latgé JP. Aspergillus fumigatus: cell wall polysaccharides, their biosynthesis and organization. Future Microbiol 2009, 4:585–595.
3. Kils FM, Ram AFJ, de Groot PWJ. A Molecular and Genomic View of the Fungal Cell Wall. In: The Mycota VIII Biology of the Fungal Cell. Edited by Howard RJ, Gow NAR. Berlin: Springer-Verlag; 2007:97–120.
4. Tefsøn B, Ram AFJ, van Die I, Routier FH. Galactofuranose in eukaryotes: aspects of biosynthesis and functional impact. Glycobiology 2012, 22:456–69.
5. Engel J, Schmalhorst PS, Dörk-Bousset T, Fernières V, Routier FH. A single UDP-galactofuranose transporter is required for galactofuranosylation in Aspergillus fumigatus. J Biol Chem 2009, 284:33859–33868.
6. Afroz S, El-Ganiny AM, Sanders DAR, Kaminsky SG. Roles of the Aspergillus nidulans UDP-galactofuranose transporter, UgtA in hyphal morphogenesis, cell wall architecture, conditioan, and drug sensitivity. Fungal Genet Biol 2011, 48:896–903.
7. Komachi Y, Hatakeyama S, Motomatsu H, Futagami T, Kizjakina K, Sobrado P, Ekino K, Takegawa K, Goto M, Nomura Y, Oka T. GfsA encodes a novel galactofuranosyltransferase involved in biosynthesis of galactofuranose antigen of O-glycan in Aspergillus nidulans and Aspergillus fumigatus. Mol Microbiol 2013, 90:1054–1071.
8. Damveld RA, Franken A, Arentshorst M, Punt PJ, Kls FM, van den Hondel CA, Ram AF. A novel screening method for cell wall mutants in Aspergillus nidulans identifies UDP-galactopyranose mutase as an important protein in fungal biosynthesis. Genetics 2008, 178:873–881.
9. Schmalhorst PS, Kappmarin S, Veneckien W, Rohde M, Müller M, Braus GH, Contreras R, Braun A, Bakker H, Routier FH. Contribution of galactofuranose to the virulence of the opportunistic pathogen Aspergillus fumigatus. Eukaryot Cell 2008, 7:1268–1277.
10. El-Ganiny AM, Sanders DAR, Kaminsky SGW. Aspergillus nidulans UDP-galactopyranose mutase, encoded by ugmA plays key roles in colony growth, hyphal morphogenesis, and conditioan. Fungal Genet Biol 2008, 45:1533–1542.
11. Heiss C, Skowrya ML, Liu H, Klutts JS, Wang Z, Williams M, Srikanta D, Beverley SM, Azadi P, Doering TL. Unusual galactofuranose modification of a capsule polysaccharide in the pathogenic yeast Cryptococcus neoformans. J Biol Chem 2013, 288:10994–11003.
12. Lee MJ, Gravetl FN, Ceronie RP, Baptista SD, Campoli PV, Choe SL, Kravtsov I, Vinogradov E, Czaczek C, Liu H, Berghuis AM, Latgé JP, Filler SG, Fontaine S, Sheppard DC. Overlapping and distinct roles of Aspergillus fumigatus UDP-glucose 4-epimerases in galactose metabolism and the synthesis of galactose-containing cell wall polysaccharides. J Biol Chem 2014, 289:1243–1256.
13. El-Ganiny AM, Sheoran I, Sanders DAR, Kaminsky SGW. Aspergillus nidulans UDP-glucose 4-epimerase UgeA has multiple roles in wall architecture, hyphal morphogenesis, and asexual development. Fungal Genet Biol 2010, 47:620–635.
14. Heesemann L, Kotz A, Echtenacher B, Broniszewska M, Routier F, Hoffmann P, Ebel F. Studies on galactofuranose-containing glycostructures of the pathogenic mold Aspergillus fumigatus. Int J Med Microbiol 2011, 310:523–530.
15. Sytnen D, Gori A, Sarfari J, Latgé JP. A new sensitive sandwich enzyme-linked immunosorbent assay to detect galactofuran in patients with invasive aspergillosis. J Clin Microbiol 1995, 33:497–500.
16. Bardalaye PC, Nordin JH. Chemical structure of the galactomannan from the cell wall of Aspergillus niger. J Biol Chem 1977, 252:2584–2591.
17. Punt PJ, Schuren FHJ, Lehmebeck J, Christensen T, Hjort C, van den Hondel CAMJ. Characterization of the Aspergillus niger PrtT, a unique regulator of extracellular protease encoding genes. Fungal Genet Biol 2008, 45:1591–1599.
18. Carvalho NDSF, Arentshorst M, Jin Kwon M, Meyer V, Ram AFJ. Expanding the ku70 toolbox for filamentous fungi: establishment of complementation vectors and recipient strains for advanced gene analyses. Appl Microbiol Biotechnol 2010, 82:1463–1473.
19. Gruben BS, Zhou M, de Vries RP. GalX regulates the D-galactose oxidoredoxuctive pathway in Aspergillus fumigatus. FEBS Lett 2012, 586:3980–3985.
20. Fekeete E, de Vries RP, Seiboth B, van Kuylk PA, Sándor E, Fekeete E, Metz B, Kubicek CP, Karaffa L. D-Galactose uptake is nonfunctional in the conidiospores of Aspergillus niger. FEMS Microbiol Lett 2012, 329:198–203.
21. Hayer K, Stratford M, Archer DB. Structural features of sugars that trigger or support conidial germination in the filamentous fungus Aspergillus niger. Appl Environ Microbiol 2013, 79:6924–6931.
22. Dalgypole SA, Ko J, Sheoran I, Kaminsky SG, Sanders DA. Elucidation of substrate specificity in Aspergillus nidulans UDP-galactose-4-epimerase. PLoS One 2013, 8:e68803.
23. Bennett JW, Lasure LL. In Mole Gene Manipulations in Fungi. San Diego: Academic Press; 1991:441–447.
24. Arentshorst M, Ram AF, Meyer V. Using non-homologous end-joining-deficient strains for functional gene analyses in filamentous fungi. Methods Mol Biol 2012, 833:133–150.
25. Meyer V, Arentshorst M, El-Ghezal A, Drevs AC, Kooistra R, van den Hondel CA, Ram AF. Highly efficient gene targeting in the Aspergillus niger kusA mutant. J Biotechnol 2007, 132:770–775.
26. Salvador LD, Suguamana T, Kitahara K, Tanoue H, Ichiki M. Monosaccharide composition of sweetpotato fiber and cell wall polysaccharides from sweetpotato, cassava, and potato analyzed by the high-performance anion exchange chromatography with pulsed amperometric detection method. J Agric Food Chem 2000, 48:3448–3454.
27. Carvalho ND, Arentshorst M, Jin Kwon M, Meyer V, Ram AF. Effects of a defective ERAD pathway on growth and heterologous protein production in Aspergillus niger. Appl Microbiol Biotechnol 2011, 89:357–373.
28. Kwon MJ, Arentshorst M, Fiedler M, de Groen FL, Punt PJ, Meyer V, Ram AF. Molecular genetic analysis of vesicular transport in Aspergillus niger reveals partial conservation of the molecular mechanism of exocytosis in fungi. Microbiology 2014, 160:316–329.

doi:10.1186/s40694-014-0006-7
Cite this article as: Park et al.: Identification of the UDP-glucose-4-epimerase required for galactofuranosylation biosynthesis and galactose metabolism in A. niger. Fungal Biology and Biotechnology 2014 1.6.