Research Article

Generation of *Aspergillus nidulans* Mutants with Deleted GDP-Mannose Transporters by Fusion PCR

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ABSTRACT

Invasive fungal infections are increasing worldwide especially in immunecompromised patients. There are few treatment options available for these life threatening infections which provoke the medical community to search for new antifungal drug targets. Fungal cell wall structure is considered one of the main differences between the human and the fungal cells. Thus its components provide excellent targets for anti-fungal drugs. Mannans are one of the important polysaccharides of the fungal cell wall, and their biosynthesis is being investigated against new antifungal targets. GDP-Mannose transporter (GMT) is one of the important enzymes in this pathway and other pathways of protein and lipid glycosylation. Fortunately, mammalian cells do not possess any GMT which makes it an excellent target for antifungals. There are many techniques to explore gene function such as gene mutation, gene tagging, promoter exchange or gene deletion. In this investigation, fusion PCR as one of the newest gene targeting procedures was used to investigate GMT biological importance. *Aspergillus nidulans nkuA* deficient strains were used to create three GDP-mannose transporter null mutants *gmtAΔ, gmtBΔ and gmtAΔ-gmtBΔ* double mutant. Although none of the tested genes was essential for viability, future work on these mutants may provide valuable information about GMT function.

Keywords: *Aspergillus nidulans*, GDP-mannose transporter (GMT), nucleotide sugar transporter (NST), Fusion PCR, gene deletion, homologous recombination

INTRODUCTION

Fungi are considered the causative agents of many diseases ranging from superficial infections to systemic infections such as invasive aspergillosis.[1,2] *Aspergillus nidulans* is usually investigated as a model organism due to its relatedness to the opportunistic human pathogen *Aspergillus fumigatus,* both species have similar sugar composition in their cell walls.[3]

Although there are wide varieties in fungal infections, such variety is not found in antifungal drugs. Moreover, echinocandins are the only group of antifungals that target fungal cell wall.[4]

Cell wall is considered the main difference between the human and fungal cells, thus it provides an excellent target for antifungal drugs.[5]

Polysaccharides (glucans, chitin and galactomannan) form 90% of filamentous fungi cell wall.[6] Galactomannans (GMs) account for 20-25% of the cell wall polysaccharides.

GMs are branched polymers of a linear core of α-mannan and β-1,5-galactofuranose side chains. The α-mannan core is formed of mannose units.[7] GDP-mannose transporter (GMT) is one of the important enzymes in mannan biosynthesis. GMT is a Golgi bound nucleotide sugar transporter (NST).

The NSTs are highly conserved trans-membrane (TM) proteins that provide link between the synthesis of nucleotide sugars (in nucleus, cytosol or ER), and the glycosylation process that occurs in the Golgi or ER lumen. Glycosylation is a critical post-translational modification that helps in generating functional proteins.[8]

GMT functions as mannose donor in the mannosylation step that produces mannans and other mannose containing glycoproteins and glycolipids.[9,10] These Mannose containing glycoconjugates play critical roles in virulence and immune response to many fungal and protozoan pathogens.[11,12] Mammalian cells do not possess any GDP-Mannose transporters, which makes GMT an excellent new target for antifungals.[13] Deletion of GDP-mannose transporter gene (*gmtA*) in *A. fumigatus* leads to absence of GMs and severe growth defects.[14] GMT has been found to be essential in *Saccharomyces, Candida* and *Aspergillus niger*.[15-17] In *A. nidulans* GMT is encoded by two genes *gmtA* and *gmtB*.[18,19]

Gene targeting is a useful technique that can be used to delete genes, to insert tags or fluorescent proteins at the ends of gene products, to replace promoters, or to replace wild-type alleles with mutant alleles. Gene targeting usually provide useful information about gene essentiality and gene function.

Historically, most gene targeting in *Aspergillus nidulans* involved transformation with plasmids which is a tedious and time consuming process.

Fusion PCR is a technique that has been developed to fuse more than two DNA sequences generating one linear construct. Fusion PCR is a very rapid technique that used for successful gene targeting in fungi including

Aspergillus.[20,21] The *A. nidulans nkuA*Δ strains are mutants that are deficient in DNA repair, which results in high frequency of accurate gene targeting during transformation.[22]

Gene deletion using Fusion PCR technique was employed in this investigation to explore the biological importance of GDP-mannose transporter (GMT) in *A. nidulans.* The fusion PCR constructs were transformed into *A. nidulans nkuA*Δ strains. These constructs induced deletion of the two genes encoding for GMT (*gmtA and gmtB*), either individually creating single mutants (*gmtAΔ* and *gmtBΔ*), or simultaneously creating a double deletion strain (*gmtAΔ - gmtBΔ)*.

MATERIALS AND METHODS

Strains, Primers and Chemicals

The strains used and generated in this study are listed in **Table 1**. *Aspergillus nidulans* strains were maintained on RPMI 1640 media supplemented with pyrimidines when needed as described by Kaminskyj.[23] The sequences of *gmtA* (AN8848.3) and *gmtB* (AN9298.3) were obtained from the Aspergillus comparative database at broad institute (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html). BLAST analysis of these gene sequences were done using Aspergillus comparative database and the hydropathy analysis was done using the TMHMM 2.0 software (http://www.cbs.dtu.dk/services/TMHMM/). The primers used in this study are listed in Table 2.

Primers were designed using Oligo Perfect primer design software (https://tools.lifetechnologies.com/content.cfm?pageid=9716&icid=fr-oligo-6?fl-oligoperfect)**.** Primers for deletion of *gmtA*, Ready Mix™ taq polymerase, RPMI1640 media, uridine and uracil were purchased from Sigma Aldrich, St Louis, MO, USA. Primers for deletion of *gmtB* and high fidelity taq polymerase were purchased from Fermentas, USA. Kits for DNA extraction, PCR purification kits, DNAase free water, dNTPs and Gene Ruler 1 kb Plus DNA ladder were purchased from Thermoscientific, Lithuania, EU. Chemicals used for preparing solutions for transformation were purchased from Alnasr chemicals Co, Abo Zaabal, Egypt.

Table 1: Characters of Strains used in this Study

A. nidulans strain	Strain characters
[1]A1148	pyrG89; pyroA4; riboB2; nkuB::*A. fumigatus*ribo B; nkuA::argB
[1]A1146	wA3; pyroA4; argB2; nkuA::argB
[2]*gmtA*Δ	pyrG89; pyroA4; riboB2; AN8848::*A. fumigatus*pyr G; nkuB::*A. fumigatus*ribo B; nkuA::argB
[2]*gmtB*Δ	wA3; pyroA4; argB2; AN9298:: *A. fumigatus pyroA*; nkuA::argB
[2]*gmtA*Δ*, gmtB*Δ	pyrG89; pyroA4; riboB2; AN8848::*A. fumigatuspyr G*; AN9298:: *A. fumigatus pyroA;* nkuB::*A. fumigatus*ribo B; nkuA::argB

[1]Obtained from Kaminskyj lab (University of Saskatchewan, Canada); [2]Generated in this study

Table 2: Primers used in this Study

Primer	Tm*	Sequence
Primers used for deletion of AN8848 (*ANgmtA*) using A*fpyrG* as marker		
gmtAup F (P1)	58.7	AGTGCCCGGATATGGTTATC
gmtAup R (P3)	60.2	AATTGCGACTTGGACGACATGATAACGGTAGCGGCGTG
gmtAdown F (P4)	60.3	GAGTATGCGGCAAGTCATGAATTGACTCGGGAGTATCCGAC
gmtA down R (P6)	60.6	CAGAACTCTTAGGTGCTTGCTTG
gmtA fusion F (P2)	62.3	ATTCTGGATGCGCGAAGTG
gmtA fusion R (P5)	61.3	TCAGACAGCCAGAATCAGGG
pyrGF (P7)	60.5	ATGTCGTCCAAGTCGCAATT
pyrGR(P8)	59.8	TCATGACTTGCCGCATACTC
Primers used for deletion of AN9298 (*gmtB*) using *pyroA* as marker		
gmtB upF (P1)	57.5	CACACCAGTTAGCAATCTTATGTT
gmtB upR (P3)	59.3	TGGTACCGTTGGAAGCCATTACGCTCAGGGGGAGAAA
gmtB downF (P4)	62.7	TGGCCAAGAGAGGATGGTAAGGCCGCAGCATCACAAG
gmtB down R (P6)	61.9	CATTTCTCAGCTAGATCTATCCGTTTC
gmtB fusion F (P2)	61	GTCGGGTAGCGCAGTTGA
gmtB fusion R (P5)	61.2	GATATTTGTATACTCTGCAACATTCGG
AfpyroAF (P7)	61.3	ATGGCTTCCAACGGTACCA
AfpyroAR (P8)	60.5	TTACCATCCTCTCTTGGCCA

Tm = Melting Temperature

Construction of the Mutant Strains

Gene deletion was performed according to the procedure described by Szewczyk et al.[20] *Aspergillus nidulans nkuAΔ* strain (A1148) was used for *gmtA* (AN8848.3) deletion, using *A. fumigatus pyrG* as selectable marker. The *A. nidulans nkuAΔ* strain (A1146) was used for *gmtB* (AN9298.3) deletion using *A. fumigatus pyroA* as a selectable marker. Both *AfpyroA* and *AfpyrG* were amplified from genomic DNA of wild type *A. fumigatus.*

Genomic DNA Extraction from Aspergillus Strains

Genomic DNA was extracted using Fermentas plant DNA extraction kit according to the manufacturer's instructions. Extracted gDNA was run on agarose gel to check the quality of DNA (by band sharpness). The gDNA was cleaned with Fermentas DNA purification kit before using it in PCR reaction.

Amplification of Selectable Markers, Upstream and Downstream of Target Genes

The primers P1 and P3 were used to amplify approximately 1Kb from upstream (5'UTR) of the target genes (*gmtA* and *gmtB*), and primers P4 and P6 were used to amplify approximately 1 Kb downstream (3'UTR) of the target genes. Both 5'UTR and 3'UTR are amplified from the *A. nidulans* gDNA. The primers P7 and P8 were used to amplify the selectable markers from the *A. fumigatus* gDNA (*pyrG* for *gmtA* deletion and *pyroA* for *gmtB* deletion) as shown in Table 2 and Figure 1a.

Each PCR reaction consists of 30 µl and had the following components: 5 µl of gDNA, 5 µl of each Primer (conc, 2µmole) and 15 µl of 2X ReadyMix™ taq polymerase. The cycling conditions include initial denaturation cycle of 94°C for 2 min followed by 30 cycles of: denaturation at 94°C for 20 sec, annealing for 30 sec (annealing temperature is usually 5°C below the primer Tm) and finally extension at 68°C for 1 min. The PCR products were run on agarose gel to check the accurate band size (Figure 3A) then purified using PCR purification kit before starting the fusion PCR step.

Generation of Linear Deletion Construct by Fusion PCR

The knockout construct generated for AN*gmtA* deletion consists of the selectable marker *A. fumigatus pyrG* flanked by predicted 5'UTR and 3' UTR for AN*gmtA* (AN8848). The fused sequences usually have overlapping ends, so primers P3 and P4 designed to be longer as they have tails. P3 has a tail similar to reverse complement of P7 (marker F) and P4 has a tail similar to the reverse complement of P8 (marker R). The primers P2 and P5 are nested primers that are used in the fusion PCR step (Figure 1b).

For fusion PCR the following components are used: DNA from the three purified PCR products (1µl each), primers (10µmole each), high fidelity Taqpolymerase (0.2 µl), 10X reaction Buffer (5 µl), and nuclease free water up to 50 µl. The cycling conditions were as follows: initial denaturation at 94°C for 2 min followed by 10 cycles of denaturation at 94°C for 20 sec, annealing step for 30 sec (annealing temp is ~5°C below the primers Tm) and extension at 68°C for 3 min (1 min/kb of the expected PCR product). Then 5 cycles of denaturation at 94°C, 20 sec, annealing for 30 sec, 68°C for 3 min extension time for the first cycle, then extension time increased for each subsequent cycle by 5 sec (as taq polymerase loses its potency). Finally 10 cycles of: denaturation at 94°C for 20 sec, annealing for 30 sec, and extension at 68°C for 3 min and 20 sec in the first cycle, then the extension time increased for each subsequent cycle by 20 sec.

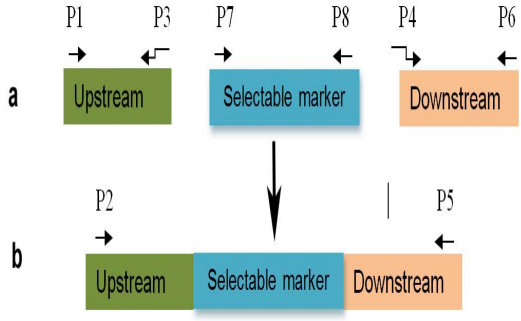

Figure 1: Generation of gene deletion construct **a)** amplification of marker and 5UTR and 3UTR of target gene by PCR, **b)** generation of linear construct by fusion PCR using nested primers P2 and P5.

Preparation of Protoplast

A. nidulans protoplast was prepared according to the procedure of Szewczyk et al.,[20] with some modifications. Briefly, 1 × 10^8 freshly harvested spores were inoculated into 20 ml RPMI medium plus uridine and uracil (for A1148) in 50 ml Erlenmeyer flask and incubated with shaking at ~150 rpm for 13–14 h at 30 °C. The hyphae were harvested by filtration through sterile filter paper. Collected hyphae were washed once with growth medium. Hyphae were re-suspended in 8 ml fresh RPMI medium in sterile 50 ml flask. Eight ml freshly prepared 2x protoplasting solution (1.28 g Vinoflow dissolved in 10 ml 0.6 M KCl and sterilized by filtration) were added and mixed by swirling. The mixture was incubated with gentle shaking (100 rpm) at 30 °C. Protoplasts are monitored microscopically and usually harvested after 2h. Undigested hyphal residues were removed by layering the protoplasting mixture gently on a sterile 1.2 M sucrose in a sterile 50 ml centrifuge tube and centrifuging at 1,800g for 10 min at 4 °C. Protoplasts were collected from the top of the sucrose solution using a sterile pipette. The collected protoplasts were placed in a sterile centrifuge tubes, mixed with an equal volume of 0.6 M KCl and centrifuged for 10 min at 1,800g. The supernatant was carefully decanted and the pellets were re-suspended in 2 ml 0.6 M KCl and transferred to micro-centrifuge tubes. The protoplasts were pelleted by centrifugation at 2,400g for 3 min, at room temperature. The supernatant was removed carefully and the pellet

was washed twice in 1 ml 0.6 M KCl. The pellet was re-suspended in 0.5 ml 0.6 M KCl, 50 mM $CaCl_2$ solution. The cells were collected by centrifuging at 2,400g for 3 min and the pellet was re-suspended in a suitable volume of 0.6 M KCl, 50 mM CaCl2 to be ready for the subsequent transformation or to be stored using the long term storage protocol.[24]

Long-term protoplast storage was following El-Ganiny et al.[24] Briefly; freshly made protoplasts were re-suspended in STC buffer (1 M sorbitol, 50 mMTris pH8, 50 mM $CaCl_2$) and adjusted to a concentration of at least 1.2 x 10^7/ml. The protoplast-STC suspension was mixed with a solution of 40 % (w/v) PEG_{4000}, at 1:4 (v/v) PEG: protoplast-STC. Dimethyl sulphoxide (DMSO) was added to a final concentration of 7 %. Aliquots of 200-300 µl protoplast-STC-DMSO suspension were frozen at -80 °C.

Transformation

The protoplast suspension was mixed with DNA construct, and incubated for 20 min in ice bath. Then it was mixed with 1 ml of 40 % (w/v) PEG_{4000}, incubated at room temperature for another 20 min, then spread on selective medium containing 1 M sucrose. When the fused constructs transformed into *A. nidulans* protoplasts, it would be inserted into the genome by homologous recombination as the upstream and downstream sequences were amplified from gDNA so they are identical to genomic sequences.

A1148 was transformed to pyrimidine prototrophy, while A1146 was transformed to pyridoxine prototrophy, creating *gmtA*Δ-*pyrG*+ strain with a wild type phenotype and *gmtB*Δ-*pyroA*+ strain with a wild type phenotype also.

To create a double mutant strain, *gmtB* gene was deleted from the *gmtA*Δ strain using *A. fumigatus pyroA* as selectable marker, producing *gmtA*Δ*gmtB*Δ double deletion strain. Conidia from the mutants were inoculated onto selective and non-selective media to assess whether *gmtA* and *gmtB* were essential genes.[25]

Confirmatory PCR (C PCR)

Spores of wild type and mutants were grown on selective media to generate mycelium for gDNA extraction. CPCR was done to compare A1148 gDNA with that from *gmtA*Δ and *gmtA*Δ-*gmtB*Δ deletion strains and A1146 gDNA with that from *gmtB*Δ deletion strains using the primers P7 (marker forward) and P6 (5`UTR reverse); the primers are described in Table 2.

RESULTS AND DISCUSSION

In silico Analysis of *gmtA* and *gmtB* Gene Sequences

The predicted amino acid sequences at the Broad Institute website showed that *GMTA* is 379 amino acids long, the gene encoding for GMTA has 1312 nucleotides, 4 exons and 3 introns. *GMTB* was 329 amino acids long, and the gene encoding for it has 1306 nucleotides, 3 exons and 2 introns. BLAST comparison of *GMTA* and *GMTB* showed that the two proteins have ~ 61 % identity. BLAST analysis of *GMTA* with Aspergillus comparative database at broad institute showed that *A. fumigatus GMTA* had 79.5 % identity with *A. nidulans* protein. BLAST analysis also showed that only *A. nidulans* and *N. fischeri* has two *gmt* genes, and also showed that *A. niger gmt* share no identity with *A. nidulans gmtA* (Table 3).

Table 3: Blasting *Aspergillus nidulans* GMTA against Aspergillus comparative database showing percent amino acid sequence identity

MO	Protein	Sequence identity (%)
A. clavatus	ACLA_009020: GDP-mannose transporter	80.8
N. fischeri	NFIA_037370: GDP-mannose transporter 1	79.3
A. terreus	ATEG_09457.1: GDP-mannose transporter	79.9
A. fumigatus	Afu5g05740: Golgi GDP-mannose transporter	79.5
A. flavus	AFL2G_10047.2: GDP-mannose transporter	79.3
A. oryzae	AO090009000688: GDP-mannose transporter	80.5
N. fischeri	NFIA_031160: GDP-mannose transporter 2	58.4
A. nidulans	ANID_09298.1: GDP-mannose transporter 2	61.7

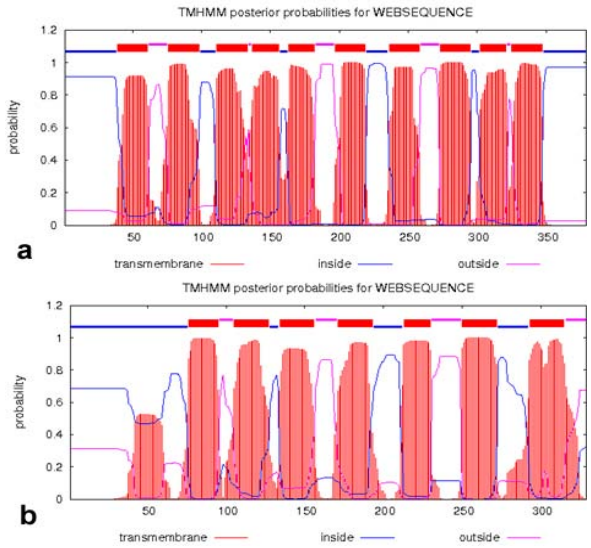

Figure 2: The hydropathy analysis using TMHMM 2.0 software showing: **a**) *GMTA* has 10 predicted trans-membrane domains and **b**) *GMTB* has 7 predicted trans-membrane domains.

The hydropathy analysis of the *GMTA*, using TMHMM 2.0 software, predicted that it has 10 trans-membrane spanning domains (Figure 2a), while the *GMTB* had 7 trans-membrane domains (Figure 2b). The results of hydropathy analysis showed that both GMT gene products are membrane associated which is compatible with the fact that GMTs are NST. Generally, NST membrane topology has been predicted to have between 6-10 trans-membrane (TM) domains linked by hydrophilic

loops on both sides of a membrane. NST topologies predicted to date suggest that most NST has an even number of TM domains.[26] A distinct exception to this is the *GMTB* in this study and the UDP-galactofuranose transporter (*UGTA*) in *A. fumigatus and A. nidulans, UGTA* has 11 predicted TM domains.[27,28] Similarly the results of Jackson-Hayes indicate that GMTs are membrane associated (Golgi localized) after tagging those genes with fluorescent proteins.[16,17]

Generation and Validation of gmtAΔ strain, gmtBΔ strain and gmtAΔ-gmtBΔ double deletion strain

To test whether *gmtA* is essential, we deleted the AN8848.3 coding sequence in the *nkuA*Δ strain A1148. Transformants were selected on RPMI 1640 agar lacking pyrimidines and containing 1 M sucrose as osmoticum. Conidia produced by primary transformants were able to germinate and sporulate when streaked on the selective medium, indicating that *A. nidulans gmtA* is not essential for viability *in vitro*. The same technique is used to test if *gmtB* is essential or not, *gmtB (*AN9298.3) coding sequence was deleted in the *nkuA*Δ strain A1146 using the *A. fumigatus pyroA* as a selectable marker. *A. nidulans gmtB* was also non-essential for viability as transformants were able to germinate and sporulate when streaked on selective medium lacking exogenous pyridoxine.

It was intended to generate a double mutant strain by mating *gmtA*Δ and *gmtB*Δ strains of different spore colored ancestors (A1148 spores are green and A1146 are white), but *gmtA*Δ spores lost its green color, and selection became impossible, so a double deletion strain was generated using the same gene targeting procedure, When *gmtB gene was* deleted in *gmtA*Δ strain to generate *gmtA*Δ-*gmtB*Δ double deletion strain, transformants were also able to grow on medium lacking both pyrimidines and pyridoxine.

To indicate that the construct replaced the target gene by homologous recombination and that ectopic integration was not contributed to the mutant's phenotype. Multiple transformation experiments are done and gave comparable results regarding the phenotype of colonies produced from primary transformants. We interpret a high level of phenotype consistency at the colony level between multiple transformants from independent experiments, as being evidence of lack of interference from ectopic integration.

To confirm that the construct integrated homologously, CPCR was performed (Figure 3 C). For *gmtA*, gDNA was extracted from parent strain (A1148) and *gmtA*Δ strains to be used as PCR template. PCR using P7 and P6 amplified ~ 2 kb band (0.9 Kb of *AfpyrG* plus the 3' UTR region) in case of the *gmtA*Δ strains, and no amplified band in case of A1148. Genomic DNA was extracted also from *Aspergillus nidulans* strain A1146 and *gmtB*Δ strains and used as template for confirmatory PCR. Af*pyroA* is predicted to be 0.9 kb. Confirmatory PCR using P7 and P6 gave only ~2 Kb band with mutant strain and no band with parent strain.

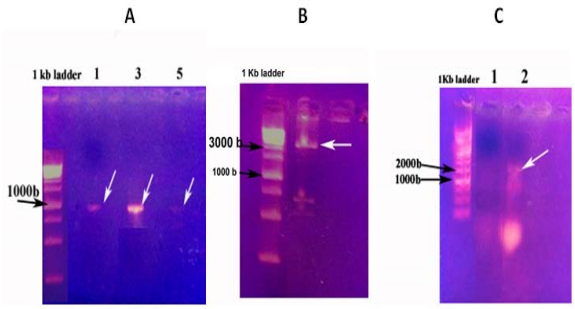

Figure 3: **A**) parts amplified using regular PCR, lane 1 → 5`UTR, lane 3 → 3`UTR and lane 5 → nutritional marker. **B**) Construct generated by fusion PCR → band of 3 Kb size. **C**) CPCR using P7 (marker F) and P6 (downstream R), lane 1 contains wild type gDNA and shows no band and lane 2 has mutant gDNA and gives1.9 Kb band (0.9Kb of marker + 1 Kb of downstream).

In this study we proved that both *gmtA* and *gmtB* are not essential for viability either when deleted individually or together.This come in accordance with the findings of Jackson-Hayes where they created a mutant in the *call11* locusin *Aspergillus nidulans* and they proved by sequencing technique that *gmtA* and *call* are identical, in *call11* strain no mutations were observed in the *gmtB* location.[18,19] Engel and his colleagues found that *A. fumigatus gmtA* also was not essential for viability.[14] The only *Aspergillus gmt* found essential was the *A. niger*,[17] and we showed by BLAST analysis that *A. niger gmt* share no similarity with both AN*gmtA* and AN*gmtB.* Regarding yeasts, *Cryptococcus neoformans* was also found to have two *gmt* genes and the *gmt1 gmt2* double mutant was also viable but exhibited severe defects in capsule synthesis and protein glycosylation.[29] Also in *S. cerevisiae* defects in synthesis of mannan-containing *N*-glycans was not lethal but causes impairment of cell integrity.[30]

CONCLUSION

In the present study null mutants were created for both *gmt* orthologs, using fusion PCR technique for gene deletion either to obtain single mutants or double mutant strains. To our knowledge this is the first double deletion strain created in *Aspergillus* for the two *gmt* genes. Although we found that both *gmt* genes are not essential for viability *in vitro*, the created mutants has defective colonial morphology which should be further investigated in future to characterize those strains and test their viability and virulence *in vivo*.

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