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Original Article

Capsule Polysaccharide Synthase 1 (CPS1) Homolog in *Aspergillus fumigatus*: A Gene Disruption Study

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Introduction: *Aspergillus fumigatus* is the leading cause of invasive aspergillosis in immunocompromised patients with a high rate of mortality. Despite introduction of several classes of antifungal drugs, the limitations of current therapies have prompted an intense research toward the discovery of new antifungal compounds. In a recent study, several potential drug targets were identified based on *in silico* comparative proteome analyses of *A. fumigatus* and *Saccharomyces cerevisiae*. A potential target, capsule polysaccharide synthase1 (CPS1) homolog gene, was identified and chosen for further study. **Methods**: The genome sequence of CPS1 homolog in *A. fumigatus* (*AfuCPS1*) was retrieved from the database and analyzed. The RT-PCR analysis was carried out to show the presence of the transcripts. A gene disruption cassette was prepared and subsequently transformed into *A. fumigatus* strain AF293. An *AfuCPS1* disruptant strain was isolated and further analyzed. **Results**: The *AfuCPS1* gene sequence was annotated, and the signal sequence and a glycosyltransferase motif were identified. The RT-PCR analysis showed that *AfuCPS1* is expressed throughout the life cycle of the fungus. The *AfuCPS1* mutant showed normal colony morphology. Antifungal susceptibility assay of the mutant using different classes of known antifungal drugs confirmed a similar susceptibility pattern to the wild-type strain. However, spore germination was affected, and a delay in germination was observed. **Conclusion**: The findings showed that the *AfuCPS1* is not essential for the normal growth of *A. fumigatus*. More investigation is underway to elucidate the physiological role of this gene in spore germination process. *J Med Microbiol Infec Dis, 2016, 4 (1-2): 25-30.*

Keywords: *Aspergillus fumigatus*, Cell wall, Gene, Spores, Aspergillosis.

INTRODUCTION

Invasive aspergillosis is a severe infectious disease in immunodeficient patients, which is mainly caused by *Aspergillus fumigatus* and is lethal unless treated [1]. Despite the relative success of current antifungal therapies, there are some deficiencies with available drugs including drug toxicity, drug/drug interactions, drug bioavailability, and drug resistance. Unfortunately, there are only a few antifungal compounds in clinical development, and some failures have been reported [2]. Hence, the introduction of new classes of antifungal drugs is urgently required.

Both classical cell-based screening methods and bioinformatics tools have been used for the discovery of new potential antifungal drug targets [3-5]. For instance, Malekzadeh *et al*. have used a computer-based comparative approach and identified some potential drug targets in *A. fumigatus* [5]. Based on their report, the whole proteome of *A. fumigatus* was screened for fungal-specific targets to avoid the human counterparts. Capsule polysaccharide synthase 1 (CPS1) homolog (*AfuCPS1*) has been identified as a fungal-specific target with possible enzyme activity (Malekzadeh S, personal communication). In the present study, we investigated the effect of *AfuCPS1* disruption on growth physiology of *A. fumigatus*. In this sense, an *AfuCPS1* disruptnat strain was generated and characterized. The primary analysis of the deletant strain demonstrated a non-essential role of the gene in this organism, while a mild delay in spore germination was observed.

MATERIAL AND METHODS

Strains, plasmids, culture conditions, and fungal transformation. A *ku80/pyrG* deficient strain of *A. fumigatus* AF293 and its parental strain were used in DNA and RNA preparations as well as minimum inhibitory concentration (MIC) assays. Gene disruption experiment was carried out in *ku80/pyrG* deficient strain. *Escherichia coli* Top10 (Invitrogen, USA) was used as the host in DNA recombinant procedures. The pGEM-T Easy cloning system (Promega, USA) was applied for cloning of PCR products.

Fungal strains were grown and maintained on sabouraud dextrose agar (SAB) or SAB agar medium containing uridine and uracil. Modified Vogel's medium [6] was used for the isolation of *pyrG*$^+$ fungal transformants. A standard polyethylene glycol-mediated transformation protocol was applied for transformation of *AfuCPS1* disruption cassette into the *A. fumigatus* protoplasts [7].

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In a phenotypic analysis of strains, radial growth rates were determined by the cultivation of fungal spores (10^4 spores) on the center of SAB and modified Vogel's agar plates at 30, 37, and 42°C, followed by the serial measurement of colonies diameter for 5 days. For germination studies, 2×10^7 fresh spores were inoculated in 1 mL SAB broth medium in 6-well culture plates and incubated at 37°C. Germination time was determined by microscopic examination of spores hourly, up to 7 h. The emergence of germ tubes was selected as a criterion for determining the germination time. All experiments were carried out in triplicates.

DNA and RNA manipulations. Fungal DNAs were extracted as previously described [8]. Total RNA was purified using a commercial kit (Qiagen, RNA easy® Mini kit, USA). All basic molecular methods were performed according to Sambrook & Russell [9]. For RT-PCR reactions, 1 µg total RNA (adjusted with NanoDrop 1000) was used in cDNA synthesis reaction (RevertAid™, Fermentas, USA). All PCRs were performed in 30 cycles of 95°C for 1 min, 58°C for 30 s, and 72°C for 30 s. As a loading control, a 600 bp actin fragment was amplified during RT-PCRs. All PCR primers used in this study are listed in table 1.

Table 1. List of primers used in this study

Primer	Sequence 5`>3`	Restriction enzyme (Underlined)
CPS1-F1	CTTAAAACTCCAGCCTAC	-----
CPS1-R1	GTTCCTCATCCCTGAGTC	----
CPS1-UP-F	GCGGGCCCCACGTCGGTATCATGCGAGA	*Apa*I
CPS1-UP-R	GAATTCTGTGCAGTCAGATGG	*Eco*RI
CPS1-DW-F	GAATTCTGCCTTGTTCTACGA	*Eco*RI
CPS1-DW-R	CTGCAGTTGGTTCCTCATCC	*Pst* I
CPS1-rt1	TGTTGAACCCTGTGTCGGTA	----
CPS1-rt2	CAACTGACGAAGCTGACCAA	----
ACT-F	ATGTCACTGTGCAGATTGTC	----
ACT-R	CGTAGAGGGAGAGAACGGCC	----

Construction of *AfuCPS*1 disruption cassette. The disruption construct was prepared as described before [10]. Briefly, a 2.9-kb genomic fragment containing the entire *AfuCPS1* coding region with upstream and downstream flanking regions was PCR amplified using primers CPS1-F1 and CPS1-R1 and subsequently cloned into the pGEM-Teasy vector. From this vector, a 900-bp 5′ flanking region of the gene was amplified using primers CPS1-UP-F and CPS1-UP-R containing *Apa*I and *Eco*RI sites, respectively. This fragment was then cloned into the *Apa*I / *Eco*RI site of the pGEM-Teasy vector. Similarly, primers CPS1-DW-F and CPS1-DW-R containing *Eco*RI and *Pst*I sites were used to amplify a 900-bp 3′ flanking region of the gene. This fragment was cloned immediately into the downstream of the 5` flanking region in *Eco*RI / *Pst*I site of the latter plasmid, and the new plasmid was named as pCPS1-UP/DW. To generate the final construct, pCPS1_KO, the *A. fumigatus pyrG* gene, was cut out from a previously prepared pGEM-pyrG plasmid using *Eco*RI and cloned into *Eco*RI site of pCPS1-UP/DW (Fig. 1).

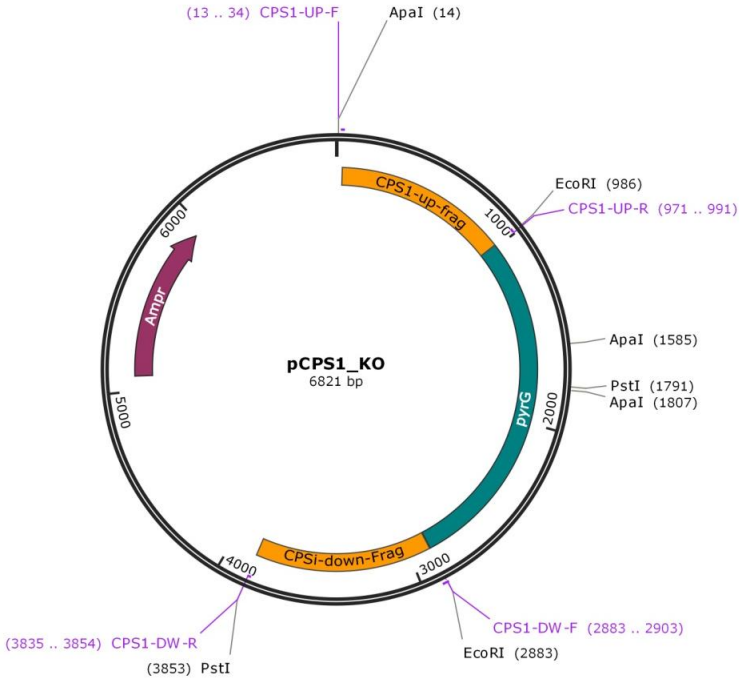

Fig. 1. Plasmid map of pCPS1_KO. The position of primers used for amplification of flanking regions of *AfuCPS1* and the main restriction enzymes used in cloning procedures are shown by the purple and black lines, respectively.

Susceptibility testing. A broth microdilution method was used to determine MICs, based on CLSI guideline M38-A2. Amphotericin B, itraconazole, hygromycin B, nystatin, calcofluor white, and voriconazole were used in susceptibility assays in a concentration range of 0-200 μgmL^{-1}. MICs were measured after 24 h for the parental strain and *AfuCPS1* mutant.

RESULTS

Sequence analysis of *CPS1* homolog (*AfuCPS1*) in *A. fumigatus.* Based on an *in silico* comparative study, the *CPS1* gene homolog was identified in *A. fumigatus* with an accession number of AFUA_8G02320 (Malekzadeh S, personal communication). This gene contains 1809 base pairs and is located on chromosome 8 (nucleotides 620482 - 622290), and the encoded product is a 602 amino-acid long protein. As a putative glycosyltransferase (GT), the protein sequence was searched in Pfam database (http://pfam.xfam.org/), and a GT motif related to the family 2 of GTs was predicted to be located at amino acids 106-352. To check whether CPS1 is a membrane-bound protein, the amino acid sequence of the protein was examined by online software TMPred (http://www.ch.embnet.org/software/TMPRED_form.html) for the presence of any transmembrane spanning regions, and the results predicted 5 transmembrane helices. Also, the prediction of the signal peptide was performed using signalP3.0 Server (http://www.cbs.dtu.dk/services/SignalP-3.0/), and a signal peptide with a possible cleavage site between amino acids 15 and 16 was predicted.

RT-PCR analysis of RNA samples from different time points of fungal growth showed the presence of *AfuCPS1* transcripts during the whole life cycle of the fungus (Fig. 2).

Disruption of *AfuCPS1* in *A. fumigatus.* An approximately 3.8-kb disruption fragment containing 5` and 3` flanking regions of *AfuCPS1* spanning the *pyrG* marker was PCR amplified using pCPS1_KO plasmid, as the template, as well as CPS1-UP-F and CPS1-DW-R primers. Subsequently, the PCR product was used in the transformation of *A. fumigatus* AF293 *ku80*$^{-}$*/pyrG*$^{-}$ strain. The positive transformants were selected on Vogel's minimum medium lacking uracil and screened by PCR to find any *AfuCPS1* disruptant strain. Considering the high frequency of homologous recombination in a *Δku80* strain of *A. fumigatus* [11], five transformants were screened by PCR using primers CPS1-UP-F and CPS1-DW-R. In 2 out of 5 transformants, a PCR product of ~3800 bp was obtained, confirming the amplification of the replacement locus instead of wild-type locus (~2900 bp product). These results indicated that *AfuCPS1* in these two transformants was disrupted (Fig. 3).

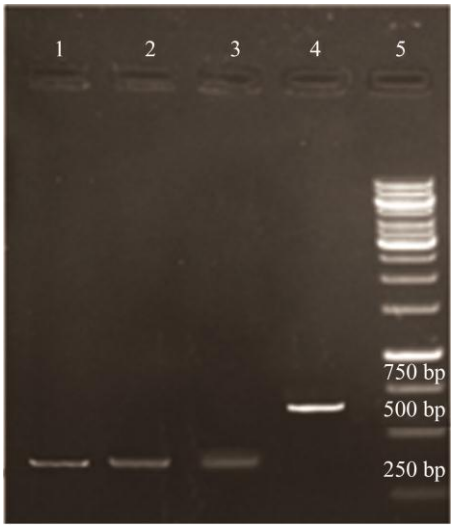

Fig. 2. RT-PCR analysis of *AfuCPS1* in *A. fumigatus*. Lane 1, RT PCR product (~270 bp) of *AfuCPS1* mRNA from *A. fumigatus* grown in SAB medium after 16 h; lane 2, after 8 h; lane 3, after 4 h; lane 4, RT-PCR product of actin transcript (~600 bp); lane 5, DNA size marker (1 kb DNA ladder, Thermo Scientific)

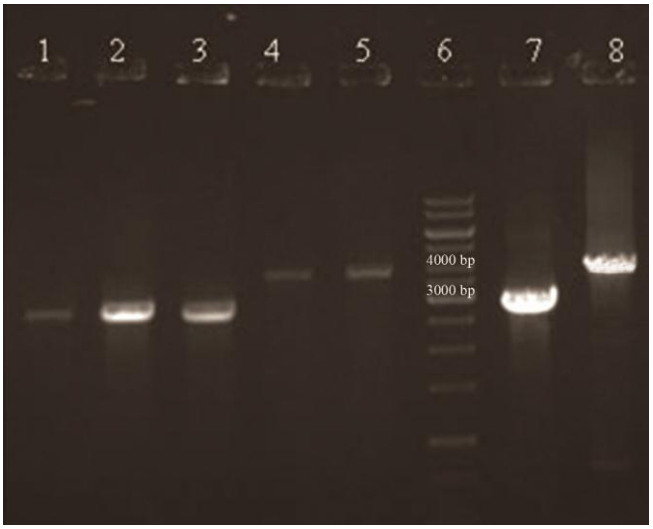

Fig. 3. PCR screening of *A. fumigatus* transformants genomic DNA using CPS1-UP-F and CPS1-DW-R primers. Lanes 1, 2, 3, *AfuCPS1* wild type locus amplified as an ~2.9 kb fragment in 3 transformants; lanes 4 and 5, the disrupted locus amplified as an ~3.8 kb fragment; lane 6, DNA size marker (1 kb DNA Ladder, Thermo Scientific); lanes 7 and 8, show amplification products of the plasmids containing the native gene and disruption construct, respectively.

One *AfuCPS1* disruptant strain was selected and further analyzed. RT-PCR analysis of this strain showed the lack of *AfuCPS1* transcripts, indicating the inactivation of the gene. In phenotypic examinations, the colony appearance and sporulation in the mutant strain were intact, and the radial growth rates of the mutant at different temperatures were similar to the wild-type strain (data not shown).

In susceptibility testing, the sensitivity of *AfuCPS1* disruptant to a range of known antifungal compounds was assessed, and the results showed no difference between the mutant and parental strain (Table 2).

In germination studies, the wild-type spores started to swell after 4 h at 37°C. The first sign of germ tube emergence appeared after 5 h in more than 90% of spores, and the

completion of germination occurred after 7 h. After this time, almost 70% of parental spores showed a long germination tube. However, in *AfuCPS1* disruptant strain, after 5 h, only 20% of mutant spores swelled, and after 6 h, only 30% of spores showed the emergence of germination tube. Overall, the complete germination of mutant spores delayed by 4 h (Fig. 4). Despite the observed delay in germination, the colony growth of mutant was not affected.

Table 2. MICs of different antifungal compounds tested on parental and mutant strains (µg/ml)

Strain	Drug					
	Nystatin	Hygromycin B	Voriconazole	Calcofluor white	Amphotericin B	Itraconazole
A. Fumigatus AF293	25	12.5	<0.1	50	1.56	<0.1
AfuCPS1 disruptant	25	12.5	<0.1	50	1.56	<0.1

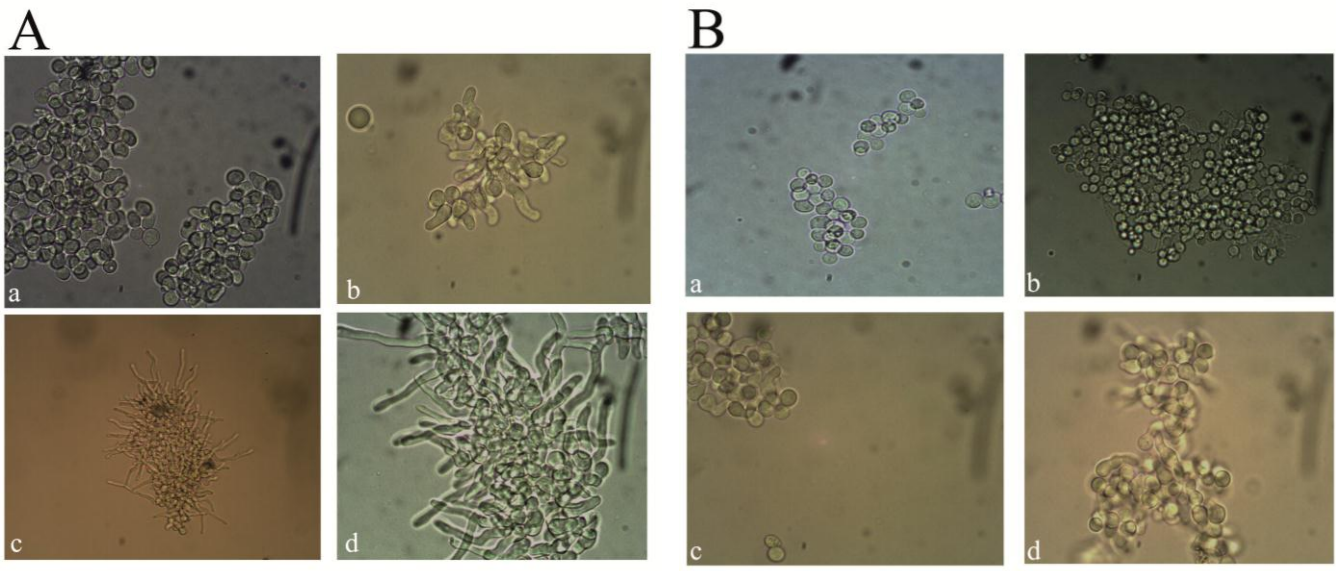

Fig. 4. Microscopical examination of germinating spores following cultivation of the wild type and *AfuCPS1* disruptant strain in SAB broth medium at 37°C. Panel A, wild type spores after 5 h (a), 6 h (b) and 7 h (c, d); panel B, *AfuCPS1* disrupted spores after 5 h (a), 6 h (b) and 7 h (c, d).

DISCUSSION

Invasive aspergillosis is estimated to affect nearly 300,000 people annually. The airborne opportunistic fungus, *A. fumigatus*, is the leading cause of invasive aspergillosis with a mortality rate of 30-50% [12]. Voriconazole, as the agent of choice, has been used successfully in the treatment of invasive aspergillosis. However, several shortcomings such as drug toxicity and the emergence of resistant isolates have diverted attentions toward the discovery of new antifungal drugs [13] [2]. Researchers have used a wide variety of methods to discover new drug targets against this pathogen. For example, fungal-specific pathways, like lysine biosynthetic pathway, branched amino acid biosynthetic pathway, and cell wall biogenesis process have been the subject of several studies [14] [15] [16]. Recently, bioinformatics methods in combination with omics data have facilitated the identification of potential targets in fungal pathogens [4] [5]. In the present study, we tried to elucidate the role of *CPS1* homolog in growth physiology of *A. fumigatus*. This potential target has been identified through a comparative proteome analysis. The amino acid sequence of the encoded protein contains a GT motif that belongs to family 2 of GTs. This family comprises a group of enzymes such as cellulose synthase, chitin synthase, mannosyltransferase, and glucosyltransferase, which mainly are involved in transferring sugar moieties. Despite the existence of sequence homology, the catalytic function of family 2 members differs widely [17]. The cell wall of fungi is a highly dynamic structure and is under constant remodeling during the fungal growth [18]. There are several remodeling enzymes including glycoside hydrolases and GTs/transglycosylases, which play a critical role in cell wall integrity and its dynamic [19]. The deletion studies on different cell wall-related transferases have led to different outcomes. For instance, while the inactivation of a glucanosyltranferase, *gel1*, did not affect the growth phenotype of *A. fumigatus*, the disruption of its homolog, *gel2*, resulted in growth and conidiation abnormalities [20]. In a recent report, Samar *et al.* [21] have identified and disrupted a putative GT, *Tft1*, in *A. fumigatus*. The deletion of *Tft1* did not result in any phenotypic change in deletant strain, indicating that the gene is not essential for fungal

survival. Similarly, the deletion of *AfuCPS1* in our study did not lead to any substantial growth defect. However, a moderate delay in conidial germination was observed. Spore germination is a complex process that is governed by various signaling pathways [22]. The cell wall plasticity is a key factor in germination process, and the altered activity of the remodeling enzymes may affect this process [23] [24]. Although we did not investigate the function of AfuCPS1 protein experimentally, based on the sequence annotation data, we hypothesized that this protein might be involved in cell wall remodeling directly or indirectly. Accordingly, the disruption of the gene can cause a delay in conidial germination as a result of altered remodeling process. Further detailed studies are required to elucidate the exact function of AfuCPS1 in *A. fumigatus*.

In conclusion, our finding showed that the *CPS1* homolog in *A. fumigatus* is a non-essential gene, but its disruption affects the germination process moderately. Although this gene cannot be considered as an appropriate drug target, the clarification of AfuCPS1 function may contribute to the better understanding of cell wall biogenesis.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest associated with this manuscript.

REFERENCES

1. Nicolle L, Rotstein C, Bourgault A, St-Germain G, Garber G; Canadian Infectious Diseases Society Invasive Fungal Registry. Invasive fungal infections in Canada from 1992 to 1994. Can J Infect Dis. 1998; 9 (6): 347-52.

2. Denning DW, Bromley MJ. How to bolster the antifungal pipeline. Science. 2015; 347 (6229): 1414-6.

3. Oliver JD, Sibley GE, Beckmann N, Dobb KS, Slater MJ, McEntee L, du Pre S, Livermore J, Bromley MJ, Wiederhold NP, Hope WW, Kennedy AJ, et al. F901318 represents a novel class of antifungal drug that inhibits dihydroorotate dehydrogenase. Proc Natl Acad Sci U S A. 2016.

4. Abadio AK, Kioshima ES, Teixeira MM, Martins NF, Maigret B, Felipe MS. Comparative genomics allowed the identification of drug targets against human fungal pathogens. BMC Genomics. 2011; 12: 75.

5. Malekzadeh S, Sardari S, Azerang P, Khorasanizadeh D, Agha Amiri S, Azizi M, Mohajerani N, Khalaj V. Identification and Evaluation of Novel Drug Targets against the Human Fungal Pathogen *Aspergillus fumigatus* with Elaboration on the Possible Role of RNA-Binding Protein. Iran Biomed J. 2017; 21 (2): 84-93.

6. Vogel H. A convenient growth medium for Neurospora (medium N). Microb Genet Bull. 1956; 13: 42-3.

7. Punt PJ, Oliver RP, Dingemanse MA, Pouwels PH, van den Hondel CA. Transformation of *Aspergillus* based on the hygromycin B resistance marker from *Escherichia coli*. Gene. 1987; 56 (1): 117-24.

8. Moller EM, Bahnweg G, Sandermann H, Geiger HH. A simple and efficient protocol for isolation of high molecular weight DNA from filamentous fungi, fruit bodies, and infected plant tissues. Nucleic Acids Res. 1992; 20 (22): 6115-6.

9. Sambrook J RD. Molecular Cloning: A laboratory manual, 3rd ed. New York: Cold Spring Harbor Laboratory Press; 2001.

10. Khalaj V, Azizi M, Enayati S, Khorasanizadeh D, Ardakani EM. NCE102 homologue in *Aspergillus fumigatus* is required for normal sporulation, not hyphal growth or pathogenesis. FEMS Microbiol Lett. 2012; 329 (2): 138-45.

11. da Silva Ferreira ME, Kress MR, Savoldi M, Goldman MH, Hartl A, Heinekamp T, Brakhage AA, Goldman GH. The akuB(KU80) mutant deficient for nonhomologous end joining is a powerful tool for analyzing pathogenicity in *Aspergillus fumigatus*. Eukaryot Cell. 2006; 5 (1): 207-11.

12. Nivoix Y, Velten M, Letscher-Bru V, Moghaddam A, Natarajan-Amé S, Fohrer C, Lioure B, Bilger K, Lutun P, Marcellin, Freys G, et al. Factors associated with overall and attributable mortality in invasive aspergillosis. Clin Infect Dis. 2008; 47 (9): 1176-84.

13. Denning DW, Bowyer P. Voriconazole resistance in Aspergillus fumigatus: should we be concerned? Clin Infect Dis. 2013; 57 (4): 521-3.

14. Schobel F, Jacobsen ID, Brock M. Evaluation of lysine biosynthesis as an antifungal drug target: biochemical characterization of Aspergillus fumigatus homocitrate synthase and virulence studies. Eukaryot Cell. 2010; 9 (6): 878-93.

15. Oliver JD, Kaye SJ, Tuckwell D, Johns AE, Macdonald DA, Livermore J, Warn PA, Birch M, Bromley MJ. The *Aspergillus fumigatus* dihydroxyacid dehydratase Ilv3A/IlvC is required for full virulence. PloS One. 2012; 7 (9): e43559.

16. Valiante V, Macheleidt J, Foge M, Brakhage AA. The Aspergillus fumigatus cell wall integrity signaling pathway: drug target, compensatory pathways, and virulence. Front Microbiol. 2015; 6: 325.

17. Breton C, Snajdrova L, Jeanneau C, Koca J, Imberty A. Structures and mechanisms of glycosyltransferases. Glycobiology. 2006; 16 (2): 29R-37R.

18. Latge JP. The cell wall: a carbohydrate armour for the fungal cell. Mol Microbiol. 2007; 66 (2): 279-90.

19. Hurtado-Guerrero R, Schuttelkopf AW, Mouyna I, Ibrahim AF, Shepherd S, Fontaine T, Latge JP, van Aalten DM. Molecular mechanisms of yeast cell wall glucan remodeling. J Biol Chem. 2009; 284 (13): 8461-9.

20. Mouyna I, Morelle W, Vai M, Monod M, Lechenne B, Fontaine T, Beauvais A, Sarfati J, Prevost MC, Henry C, Latge JP. Deletion of GEL2 encoding for a beta(1-3)glucanosyltransferase affects morphogenesis and virulence in *Aspergillus fumigatus*. Mol Microbiol. 2005; 56 (6): 1675-88.

21. Samar D, Kieler JB, Klutts JS. Identification and deletion of Tft1, a predicted glycosyltransferase necessary for cell wall beta-1,3;1,4-glucan synthesis in *Aspergillus fumigatus*. PloS One. 2015; 10 (2): e0117336.

22. Osherov N, May GS. The molecular mechanisms of conidial germination. FEMS Microbiol lett. 2001; 199 (2): 153-60.

23. Bouzarelou D, Billini M, Roumelioti K, Sophianopoulou V. EglD, a putative endoglucanase, with an expansin like domain is localized in the conidial cell wall of *Aspergillus nidulans*. Fungal Gen Biol. 2008; 45 (6): 839-50.

24. Takaya N, Yamazaki D, Horiuchi H, Ohta A, Takagi M. Cloning and characterization of a chitinase-encoding gene (chiA) from *Aspergillus nidulans*, disruption of which decreases germination frequency and hyphal growth. Biosci Biotechnol Biochem. 1998; 62 (1): 60-5.