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Knocking out the *pptA* gene in *Aspergillus fumigatus* could result in new protein profile and promising targets for antifungals

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ABSTRACT

The filamentous fungus *Aspergillus fumigatus* is the most considerable common opportunistic pathogen. This is due to the prevalence of its occurrence in patients with immune debilities. It can cause diseases with impact extends to life-threatening seriousness. This fungus recently showed interesting means of adaptation, biofilm formation, and morphogenesis within the host biome. Its ability to invade and systematically disperse in the patient's body is of great concern. The complications of antifungal therapy and the limited target available to treat such infections, urge intensive and thorough research to uncover new more effective targets. The present study revealed a novel possibility of making the *pptA* an effective address in the fungus for antifungal treatment. Knocking-out the *pptA* gene resulted in halting pathogenecity as it correlated with essential genes of pathogenecity *medA* which showed considerable down regulation. Significant increase of protein profile has been discovered in Δ*pptA* strains as *UBC* gene highly activated and protein assay revealed high rate of production. In conclusion, the present study emphasize *pptA* as a possible strong antifungal target and declares high protein output from Δ*pptA* which may be a promised strain for industrial and beneficial protein production.

1. INTRODUCTION

Annually, there are reports of deaths of around 2 million people in the world due to fungal infections (Brown *et al.*, 2012). Such ratio of death is even beyond the mortality rate of malaria (Vos *et al.*, 2012). The most encountered and considered infections by a filamentous fungus are related to the *Aspergillus fumigates* (*A. fumigatus*) (Hospenthal, *et al*, 1998). It can cause life threatening invasion in patients. Vast majority of infections with *Aspergillus fumigates* is ended up with death (Gupta *et al*, 2012). This is specifically in patients with immune system debility. Immune system may be debilitated due to many reasons, such as Acquired immunodeficiency Syndrome (AIDS), leukemia, transplantation, and chemotherapy receiving patients (Brown *et al.*, 2012). The fungus has the ability to produce high rate of conidia. These spore structures are then spread through air into the environment (Filler and Sheppard, 2006). Human without noticing may inhale more than

hundred of these spores a day while breathing, specifically in areas contaminated with such conidia. These small tiny structures can easily penetrate the air passages into the alveoli of the lung (Chandrasekar *et al*, 2000). However, in normal circumstances as the body defense system working, it can wipe out these spores through the innate and acquired immune army. Nevertheless, for patients who suffer immune system debility or suppression the presence of those spores are problematic, as failure to eradicate those conidia may eventually lead to germination and growth of the *Aspergillus fumigatus* (Ben-Ami *et al*, 2010). This can cause tissue invasion and aspergillosis may arise. Aspergillosis is one of the most infective life threatening diseases in those patients. The germination and growth of the fungus inside the human host are mostly dependent on the proteins and metabolic activities of the fungus. These pathways of pathogenecity and invasiveness are based on the active gene expression of the fungus to survive and nourish in such a complex new environment (Dagenais and Keller, 2009). The onset of the infection and success of the pathogen are of many types. Patients with previous history of asthma or fibrosis can show allergic response known as Allergic Brnonchopulmonary Aspergillosis (ABPA). Also fungal sensitization may occur in asthma patients. Coughing, sneezing, running nose, and hay fever like symptoms may appear. The success of germination may cause long persistence of the fungi in the chest known as Chronic Pulmonary Aspergillosis (CPA). Patients with underlying lung diseases such as tuberculosis are more prone to such infection and complications. Aspergillus can grow in pre-existing cavities of the lung and form a ball like structure of hyphae known as (Fungus Ball) (Denning *et al*, 2009).The fungus ball in addition to the mycelia it may contain necrotic tissues, mucus and immune responded cells. Patients may suffer from fatigue, muscle pain, long term coughing and difficulty of breath. The most dangerous and life-threatening type of infection is invasive aspergillosis (IA). Invasive aspergillosis is of a great medical concern as it can cause high mortality rate in patients with critically ill conditions in addition to the immunocompromised individuals (Denning *et al*, 2003). None specific symptoms may appear such as fever, pain of the chest and production of mucus sputum. Treatment of fungal infections and especially aspergillosis, so far, has not succeeded in limitation of the high mortality rate (Kousha *et al*, 2011). The invasive systematic infections by *Aspergillus fumigatus* and other fungi require systematic interference with highly affective antifungal (Walsh *et al*, 2004). This is in order to deliver the drug to the targeted area in deep tissue infections. Intravenous and oral administrations of systematic antifungal are used widely nowadays in order to eradicate the infection. However, the emergence of antifungal resistance and the lack of variety of targets declined the success possibilities of cure. In addition, the fate of the antifungal and their biochemical activity in the tissues also has been of medical mycology concern (Denning and Bromley, 2015). Consequently, researches are ongoing toward the discovery of new antifungal targets with more potential treatment bioactivity. Billions of dollars annually been spent for the available antifungals in the market. These are includes echinocandins, azoles, polyenes and pyrimidine analogues, but however the death rate are still high. These classes have fenced ability to attack fungal cell rather than chitin and sterols (Denning and Hope, 2010). Thus, finding a new target for antifungals with high

specificity, vital essentiality and accessible feature is a goal of the present study. The target, however, should be crucial for biological activity of the pathogen. The Phosphopantetheinyl transferases (PPTase) is shown to be a fundamental metabolite in many bacteria as well as plant fungal pathogens (Walsh *et al*, 1997). With little investigation of this imperative target in human pathogens, the current study shed lights on the metabolites and protein profiles of *pptA* gene and its possibility to be an antifungal target. Phosphopantetheinyl transferases (PPTase) is a necessary enzyme for the adjustment of many other proteins after translation such as polyketide synthases, nonribosomal peptide syntheases, and fatty acid synthases.

2. MATERIALS AND METHODS:

2.1. Growth of fungus

The ATCC3626 strain of *Aspergillus fumigatus* used to be grwon under standard condition on Potato Dextrose Agar PDA (Temperature: 24°C to 26°C Atmosphere: Typical aerobic).

2.2. DNA Extraction:

Slightly tuned and optimized Cetyl Trimethyl Ammonium Bromide (CTAB) method for DNA extraction was used as described by Fraczek *et al* 2013. Where spores were collected using surface wash out with PBS/Tween20 and centrifuged at 14000 rpm for about 2 minutes. The sediment was then added for a suspension of the extraction buffer of CTAB. The sediment then added to a 2mL screw capped tubes that previously filled up to 300µL with sterile glass beads. Respective three cycles of vortexing were then applied intervened by 10 minutes incubation of the tubes at 60˚C. The tubes then left for rest at room temperature 5 minutes and centrifuged later at 1400 rpm for 5 minutes. Amount of 700µL of the supernatant transferred to sterile 2mL tubes and 4µL of RNase (Qiagen) to be added. The tubes were incubated at 37˚C for about 10 minutes. Using 25:1 chloroform:isoamyl alcohol to eliminate any protein content, amount of 700µL and mix by vortexing for 10 seconds only. Centrifugation of the tubes then performed with 1400 rpm for 2 minutes. An amount of 600µL of the supernatant then added to a set of new sterile 1.5mL tubes. Isopropanol was added to the tubes and mixed manually by inverting the tubes up and down. Another round of centrifugation for 5 minutes at 14000 rpm was performed and the supernatant discarded. Ethanol with 70% concentration were added (500µL) and vortexed for only 5 seconds in order to wash the sediment from any debris and proteins. The mixture centrifuged again at 9000 rpm for 2 minutes. The ethanol supernatant removed and the pellet let to dry at room temperature. DNA were harvested by adding 100µL of molecular grade dH2O.

2.3. Primers:

The primers were designed, optimized and efficiency test performed. Table 2.1 shows the list of primers used in the present study.

Table 2.1. List of primers used in this study (Johns, 2015)

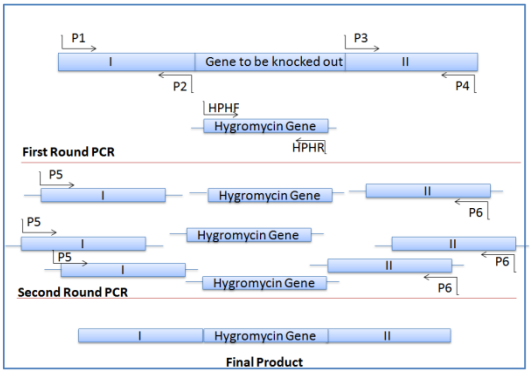
Gene	Sequences
pptA_P1	CCGGTCTCTTTCTCTGCATC
pptA_P2	TAGTTCTGTTACCGAGCCGGTC AAACGAGGGAGGAGTCAG
pptA_P3	GCTCTGAACGATATGCTCCCC CATGCAATATTCCACAGGA
pptA_P4	CGGCGTACAGTTCGACATTA
pptA_P5	CGTCCACCTGGATACCTTGT

pptA_P6	TCTTCATTGGCAACCATCAG
pptAF	ACCACCTCAGGGACAGACAC
pptAR	CTCCTTGAGAGCCCAGTACG
HPHF	CCGGCTCGGTAACAGAACTAA CGGCGTAACCAAAAGTCAC
HPHR	GGGAGCATATCGTTCAGAGCT CTTGACGACCGTTGATCTG
*medA*F	CGTTACCCAACTTAATCGCCTT GTATTCATTACCCGACCCTTCC
*medA*R	CGAAACGACGTAGATGAAAGA
*UBC*F	ACCAGCAGAGGCTGATCTTT
*UBC*R	ACCTCTGAGGCGAAGGACTA

medA and *UBC* are designed specifically for this study.

2.4 Generation of Knockouts in *A. fumigatus*:

The methodology for generating a knockout strain of *A. fumigatus* through PCR fusion along with transformation of the fungus has been well addressed by Berl Oakley's group (Szewczyk, 2006).



A

B

Figure 1: A: The fusion PCR technique for knocking out the gene of interest (adapted from Szewczyk *et al.*, 2006). Both flank ends along with a selected marker were amplified in the first round of PCR reaction. The second round included nested primers P5 and P6 to increase the precision of product amplification.
B: Generation of knowckout sequence after 2 cycles of fusion pcr resulted in 4kb DNA sequence.

2.5. Growth rate determination:

The growth rate of both radial on agar and in broth were determined using Vogel`s Minimal Media (VMM). However in order to harmonize the growth rate of both knockout and parental strains, 0.5 mM FeSO4 or 10 mM lysine or both 0.5 mM FeSO4 and 10 mM lysine has been added (Fraczek *et al* 2013).

2.6. The Bradford (Coomassie) protein production assay:

The amount of $2x10^6$ harvested spores of knockout strain and parental strain of *A. fumigates* of were added to 250 ml broth VMM in flasks. The negative control was established as media only. The samples were each of 5 replicates. Shaker incubator was used at 200 rpm. From the supernatant of the growth medium, one millilitre were taken and sterilized by filtration using Whatman #2 filter paper. The Bradford assay (Coomassie) protein detection kit were used (thermo scientific). The protein

estimation were performed as per the manufacturer protocol. The replicates of samples were added to microplates and their absorbance were determined using scanning spectrophotometer (Bio TEK Synergy). A T-Test used to indicate significance of protein production.

2.7. Real- Time PCR:

Gene expression for the knockout strain along with parental strains was compared by using the development modification gene *medA* gene along with the ubiquitin C *UBC* in both. The gene expression rates were determined using the real-time PCR (BIO-RAD). The data were analysed using REST2000 program.

2.8. Statistical Analysis:

The T-test paired group analysis were used to determine difference between parental (WT) and knockout (KO) strain of *A. fumigatus*. Prism graphpad were used to present the data and calculate correlations. REST2000 program were used to analyse data of the real-time PCR.

Results and Discussion:

The present study aimed to generate a knock-out strain of *A. fumigatus* that lacks the pptA gene. This is as a novel step toward finding new targets for the antifungal used against this filamentous invasive fungus. The results showed interesting approaches when parental wild type compared to the knockout strain. The growth rate and the protein profile showed promising results toward further investigation into this essential gene and seeking vital antifungal target. Generation of the knockout strain successfully performed (Figure 2) and the strain showed effective growth on media supplemented with nutrients such as $FeSO_4$ and Lysin (Figure 3).

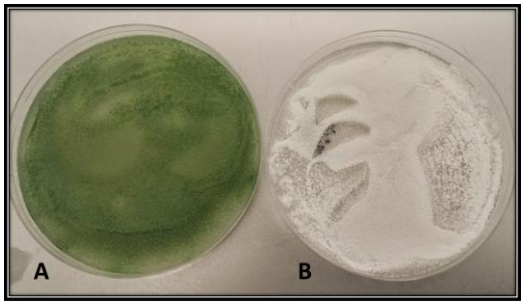


Figure 2: The two tested strains of parental (A) and the Δ*pptA* (B).

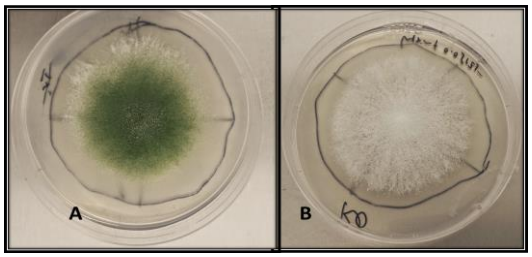


Figure 3: Growth rate determined through radial growth diameter determination of both parental strain (A) and the knockout strain (B). The knockout strain was supplemented with 1.5 mM FeSO4 and 10 mM lysine.

Growth on broth medium were also determined and tuned in order to get the optimal equal growth of both strains. However providing 1.5 mM FeSO4 and 10 mM lysine were also necessary for the growth of the knockout in broth medium to come in parallel with the wild type strain (Figure 4)

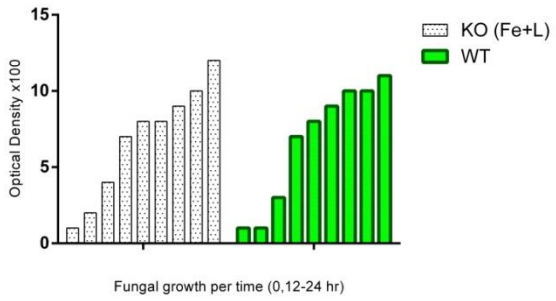


Figure 4: Growth rate determined through optical density determination of both parental strain (WT) and the knockout strain (KO). The knockout strain was

supplemented with 1.5 mM FeSO4 and 10 mM lysine.

This indicated that the deletion of the *pptA* gene urges the fungi for the need of lysine and ferrous. Thus essentiality of the *pptA* for functions related to the cellular virulence as ferrous is strongly needed to activate set of genes that have crucial role in adaptation, ergosterol biosynthesis, and drug resistance (Haas, 2012). Lysine was shown to be a possible antifungal target. Depletion of lysine resulted in activated lysine uptake by the fungus (Schöbel *et al*, 2010). The result of this study shows possibility of targeting *pptA* products to starve the fungal cell from both iron and lysine.

The protein production assay for both strains showed interesting profile for the knockout. Compensation of the depleted and deleted enzymes results in the high production of various proteins (Figure 5). The results of the present study showed significant increase of protein production profile of the knockout strain, specifically when the media supplemented with 1% of Carboxymethyle cellulose (CMC) and 1% Glycerol.

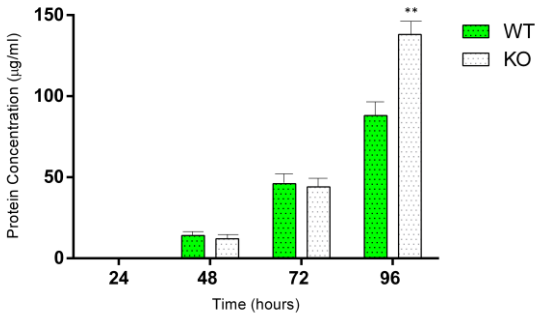


Figure 5: Bradford (Coomassie) protein assay. The total protein outputs of the knockout strain have been increased significantly by time.

Increase of the protein production is a promising approach toward further study of the *pptA* mutant. Seeking essential proteins and their reliability on each other is a considerable concern. The protein production tested for the knockout strain through a CMC contained medium. It turned out that the knockout strain have the ability to assimilate cellulose effectively and significantly more than the wild type (Figure 6).

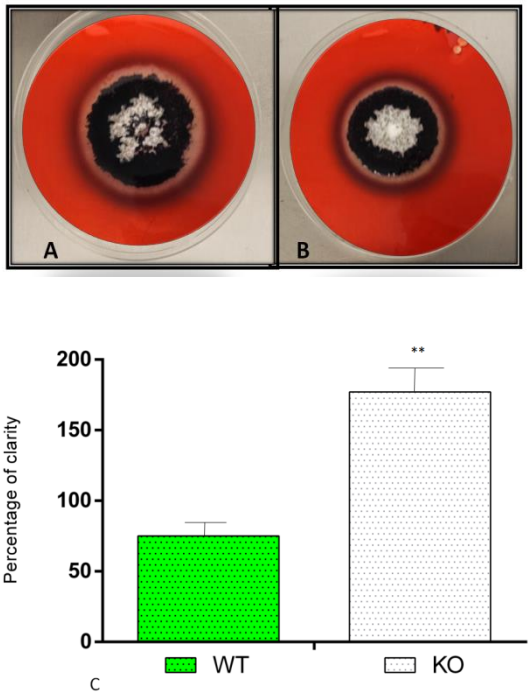


Figure 6: Growth on CMC contained medium showed significant radial progress of the knockout strain (A) in compare to the parental strain (B). The statistical analysis showed significant differences of the percentage of clearance of cellulose (C).

From the protein production assay and the growth characteristics, the study showed that the knockout strain can present high protein yield. This is in order to compensate the secondary metabolites that will be missing when the gene is disrupted, indicating the essentiality and crucial role of the *pptA* gene in the fungal growth and progression. The new protein profile can also provide closer insight for the ability of the organism to produce some new industrial proteins that can be

harvested. Cellulose assimilation test indicated high rate of cellulase enzyme providing a promising approach toward the capability of the fungi to be directed for industrial production of cellulase enzyme.

Results of gene expression and transcription profile for selected genes of pathogenecity and ubiquitin protein activity have revealed an interesting up-regulation of the genes when *pptA* has been knocked out in compare with the parental strains. Table 2 shows the real time PCR interpretation result. The *medA* gene was down-regulated by 3 folds while *UBC* is actively expressed with 10 folds expression increase.

The medA gene is shown to have a great role in pathogenecity and adaptation of A. fumigatus. It has roles in morphogenesis and hyphal growth. Recently the gene is indicated a high efficiency in fungus ball formation and biofilm triggering (Busby *et al*., 1996, Yu *et al*., 2006). In a comprehensive study of Twumasi-Boateng and others (2009), *medA* shown to be one of the most effective genes contributed to virulence.

Table 2: The real time PCR results of gene expression for transcription profile of selected genes.

Gene	Type	Reaction Efficiency	Express	P	Result
medA	TRG	0.99	3.077	0.014	Down
UBC	TRG	0.99	10.76	0.012	UP
		medA is UP-regulated in sample group (in comparison to control group) by a mean factor of 3.077 (S.E. range is 1.27 – 1.32).			
Interpretation		*UBC* is UP-regulated in sample group (in comparison to control group) by a mean factor of 10.766 (S.E. range is 1.73 - 1.88).			

Thus, the down-regulation of *medA* in Δ*pptA* indicates correlation of the gene with the pathogenecity, which eventually present it as a promising novel antifungal target. On the other hand, *UBC* up-regulation may reveal the response of the cell toward high protein production profile as indicated in the present study when *pptA* is knocked out.

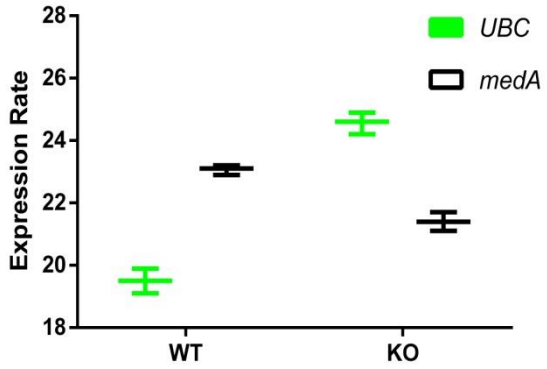


Figure 7: The transcriptomic profile of the selected genes expression levels of both wild type (WT) and knockout strains (KO) in a mixed biofilm.

The *UBC* is shown to play a role in pathogenecity and virulence of *A. fumigatus* as an important step of successful adhesion and initiation of disease related to endothelial response of the host (Richie *et al*., 2011). Also in other studies has been used as housekeeping gene for gene expression studies (Ren *et al.,* 2010).). In fact protein elimination and cellular protein level regulation are robust functions of the *UBC* (Shankar *et al.,* 2005). The current findings of this study showed interesting response of *UBC* gene expression when protein levels is increasing. This correlating the high rate of protein with gene expression changes in the knockout strain of the pathogenic *A. fumigatus*. Nevertheless, such a high input of proteins may pave the way toward finding new antifungal targets as well as beneficial proteins and

metabolites that can be industrially produced.

3. Conclusion:

In conclusion, the present study showed that the gene *pptA* is strongly related to pathogenecity of *A. fumigatus*. This is in addition to its vital role in essential cellular mechanisms of growth and protein production control. The deletion of this gene could reveal a new protein profile of which further study is crucial to identify the possibility of industrial and production harvest of those proteins.

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