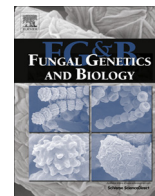




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The newly nonsporulated characterization of an *Aspergillus fumigatus* isolate from an immunocompetent patient and its clinic indication

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ABSTRACT

Aspergillus fumigatus (*A. fumigatus*) commonly produces abundant and heavily melanized infectious conidia, which are the primary agents that cause invasive aspergillosis (IA) in immunocompromised patients. We isolated a white nonsporulating *A. fumigatus* strain (*A_{1j}*) from an immunocompetent patient. It was identified by histopathological examination and morphological observation, and subsequently confirmed by DNA sequencing of internal transcribed spacer (ITS) regions and partial β -tubulin genes. Neither a long waiting time nor passage on various medium types could stimulate the formation of spores and pigment. No significant relative difference was found in sensitivity to antifungal agents or cell wall destabilizing reagents, as compared to wild-type *A. fumigatus* Af293. Nevertheless, *A_{1j}* was hypovirulent in the immunosuppressed mice model, consistent with the good result in our patient. RNA deep-sequencing analysis (RNA-seq) revealed that hundreds of transcripts were significantly dysregulated, including those related to pigmentation and sporulation. qRT-PCR confirmed the anergic state of key regulator *brlA* for sporulation under the induction of conidiation conditions, but without mutation. To the best of our knowledge, this is the first report of a white, nonsporulating *A. fumigatus* strain infection in an immunocompetent patient. In our opinion, *A_{1j}* may represent a mutant of typical *A. fumigatus*, providing a new clue for identification of clinical *A. fumigatus* isolates. Furthermore, the good prognosis of our patient and the reduced virulence in the mice model infected with *A_{1j}* highlight the potential of sporulation inhibitors as a new generation of antifungal agents.

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1. Introduction

A. fumigatus, a ubiquitous mold, releases numerous asexual spores (conidia) into the atmosphere. These conidia are the vehicles for dispersal and survival; they are also the initial source of inocula for infection (Upadhyay et al., 2013). Under favorable conditions, conidia can break dormancy and grow as elongated and highly polarized hyaline hyphae. Upon achieving competence and exposure to air, hyphae produce aerial conidiophore stalks and develop elaborate multicellular conidiophores that generate chains of melanized uninucleate conidia (Upadhyay et al., 2013). *A. fumigatus* conidia are small enough (2–3 μ m in diameter) to reach

the pulmonary alveoli after they have been inhaled (Latge, 1999). Once the conidia reach the alveoli, they swell and germinate, producing hyphae that invade the pulmonary parenchyma (Bodey and Vartivarian, 1989). Pulmonary diseases caused by *A. fumigatus* are often accompanied with a spectrum of clinical syndromes (Soubani and Chandrasekar, 2002). Invasive aspergillosis (IA) is primarily found in severely immunocompromised patients, critically ill patients, or those with chronic pneumonitis, such as chronic obstructive pulmonary disease (COPD). Chronic necrotising aspergillosis (CNA) is a locally invasive infection and is mainly seen in patients with mild immunodeficiency or with a chronic lung disease. Aspergilloma and allergic bronchopulmonary aspergillosis (ABPA) are noninvasive forms of *Aspergillus* lung disease. Aspergilloma is a fungus ball that develops in a pre-existing cavity within the lung parenchyma, whereas ABPA is a hypersensitivity manifestation in the lung that almost always affects patients with asthma or cystic fibrosis (Zmeili and Soubani, 2007).

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BrlA, a C₂H₂ zinc finger transcription factor, is the master regulator that controls the initiation of conidiophore development in *A. fumigatus*. For sporulation to progress properly, AbaA and WetA, two other important regulators that function downstream of BrlA, are required. The *abaA* gene is activated during the middle stage of conidiophore development; the *wetA* gene is required for the activation of late conidiation-specific genes. These three downstream genes act in concert with other genes to control conidiation-specific gene expression and determine the order of gene activation during conidiophore development and spore maturation (Mah and Yu, 2006; Tao and Yu, 2011).

Melanin is an amorphous polymer that is produced by a variety of microbes and higher eukaryotes. It not only helps fungi to fend off various environmental insults (e.g., UV irradiation) but also helps with animal and plant pathogens' survival in hosts (Upadhyay et al., 2013). 1,8-dihydroxynaphthalene (DHN) melanin and pyomelanin are the two major types of melanin synthesized by *A. fumigatus* (Bayry et al., 2014). The DHN-melanin is synthesized *de novo* using endogenous substrates through the DHN intermediate. The final product is produced by a series of enzymatic reactions involving six enzymes encoded by a six-gene cluster (Jahn et al., 1997; Tsai et al., 1997, 1998, 1999, 2001; Langfelder et al., 1998; Fujii et al., 2004). DHN-melanin adheres to the cell wall of *A. fumigatus* conidia and gives them their characteristic grey-green color (Youngchim et al., 2004). Another melanin is called pyomelanin, and is generated when L-tyrosine or L-phenylalanine presents in the medium. *A. fumigatus* can produce pyomelanin with an intermediate (homogentisate, HGA) of the tyrosine degradation pathway, which is controlled by another six-gene cluster (Schmaler-Ripcke et al., 2009; Keller et al., 2011). Pyomelanin appears to bind to the surface of hyphae and excrete into the medium (Schmaler-Ripcke et al., 2009). Both melanins protect the fungus against reactive oxygen species produced by host immune cells, whereas DHN-melanin alone was found to be related to *A. fumigatus* virulence in mice infection models (Langfelder et al., 1998; Tsai et al., 1998; Latge, 1999; Jahn et al., 2002; Luther et al., 2007; Schmaler-Ripcke et al., 2009; Keller et al., 2011; Buskirk et al., 2014).

Members of *Aspergillus* section *Fumigati* have been reported to be variable species (Katz et al., 2005). So far, there are 51 species in the section *Fumigati*, which includes *A. fumigatus*. Among them, 12 are clinically relevant species, and *A. fumigatus* is the most frequent agent of IA followed by *Aspergillus lentulus* and *Aspergillus viridinutans* (Sugui et al., 2014). Many fungi produce similar hyphal structures—that is, septate hyphae with acute-angled branching, a morphological description that has been classically used to characterize colonization with *Aspergillus* species (Khare et al., 2014). These species may be misidentified as *A. fumigatus* if we only rely on microscopic morphology. Previous molecular studies have uncovered genetic distinctions among several phenotypically identified *A. fumigatus* isolates, such as *A. lentulus*, *A. viridinutans*, *Aspergillus felis*, *Aspergillus fumigati*affinis, *Aspergillus fumisynnematus*, *Aspergillus udagawae*, *Neosartorya fischeri*, *Neosartorya hiratsukae*, *Neosartorya pseudofischeri*, and *Neosartorya udagawae* (Balajee et al., 2006; Serrano et al., 2011; Swilaiman et al., 2013; Khare et al., 2014). Some of these species have been described as human pathogens, particularly *A. lentulus*, *A. viridinutans*, and *A. felis*, which resemble *A. fumigatus* and tend to be refractory to antifungal treatment and prone to produce chronic infection when compared with *A. fumigatus* (Vinh et al., 2009; Zbinden et al., 2012; Barrs et al., 2013; Alvarez-Perez et al., 2014). Therefore, accurate and early species identification with molecular diagnosis is of great importance for disease management.

In this study, we isolated and identified a white nonsporulating *A. fumigatus* strain A_{ij} from an immunocompetent patient. We compared it with the reference strain Af293 in terms of phenotypic

characterization, sensitivity to antifungal agents and cell wall destabilizing reagents, virulence in immunosuppressed mice model, and difference in transcriptome profiles. Although both strains had similar reactions to chemical reagents, there were some significant differences in other aspects. The white velvety colonies of A_{ij} could passage stably, regardless of incubation period or medium selected for inoculation. Despite great differences in morphogenesis and transcriptome profiles from the reference Af293, A_{ij} was unequivocally identified as *A. fumigatus*. Because the differences between A_{ij} and Af293 were so significant in terms of clinical prognosis and virulence of mice infection model, a potential novel antifungal drug candidate may be possible.

2. Materials and methods

2.1. Fungal strains and media

A. fumigatus wild-type strain Af293 (purchased from FGSC, Fungal Genetics Stock Center, School of Biological Sciences, University of Missouri, Kansas City, Missouri, USA) was used as the reference strain in this study. The clinical strain was isolated from a 76-year-old female patient with pulmonary aspergillosis (PA), stored in CMCC (China Center for Medical Microorganisms Culture Collection, Institute of Dermatology, Chinese Academy of Medical Sciences and Peking Union Medical College, Nanjing, China), and named A_{ij}. The strains were propagated on solid (or in liquid) YAG medium, according to Zhong et al. (2012). Other media used in this study included Potato Dextrose Agar (PDA), Czapek Dox Agar (CDA), Sabouraud's Agar (SDA), Yeast Peptone Dextrose Agar (YPD), Czapek Medium (CA), CAY (CA + 0.5% yeast extract), and Malt Extract Agar (MEA), as previously described (Balajee et al., 2005; Paul and Moye-Rowley, 2013).

2.2. Microscopy

The slide culture method for microscopy assay was performed as previously described with a few modifications (Fujita, 2013). Briefly, several blocks of YAG agar medium (about 50–100 mm²) were placed on a YAG agar plate, and YAG blocks that had been inoculated with the corresponding strain were overlaid with square coverslips (18 × 18 mm). Strains in this set were incubated at 37 °C for 18 h or longer prior to observation under microscope. Each fungus was observed by placing the coverslip on a glass slide. Differential interference contrast (DIC) images of the morphogenesis were collected with a Zeiss Axio Imager A1 microscope (Zeiss, Jena, Germany).

2.3. Plate assay

Voriconazole (VRC, Sigma-Aldrich), itraconazole (ITC, Sigma-Aldrich), and amphotericin B (AMB, Amresco) were diluted in DMSO; congo red (CR) and sodium dodecyl sulfate (SDS) were in sterile deionized H₂O. To test the antifungal reagent sensitivity, YAG agar plates covered by fungal hyphae were perforated with a small perforator (5 mm in diameter), and then the fungal blocks were inoculated upside down in the center of each fresh YAG agar medium containing 2, 4, 8, 16, 32 µg/ml ITC; 0.125, 0.25, 0.5, 1, 2 µg/ml VRC; 4, 8, 16, 32, 64 µg/ml AMB; 75, 150, 300 µg/ml CR; or 0.008%, 0.01%, 0.02% SDS, respectively. For perforated Af293 plates, 100 µl spore suspensions (10⁸/ml) were spread well on the YAG agar plates, and incubated at 37 °C for 15 h. Meanwhile, A_{ij} was inoculated using a vaccination needle, and incubated at the same temperature for 2 d. Thus, both strains were hyphal appearance before perforation. The reagent plates were photographed after incubation at 37 °C for 3 d, and the diameter of

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