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AreA controls nitrogen source utilisation during both growth programs of the dimorphic fungus *Penicillium marneffei*

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ABSTRACT

The opportunistic pathogen *Penicillium marneffei* displays a temperature-dependent dimorphic switching program with saprophytic hyphal growth at 25 °C and yeast growth at 37 °C. The *areA* gene of *P. marneffei* has been isolated and found to be required for the utilisation of nonpreferred nitrogen sources during both growth programs of *P. marneffei*, albeit to differing degrees. Based on this functional characterisation and high degree of sequence conservation with other fungal GATA factors, *P. marneffei areA* represents an orthologue of *Aspergillus nidulans areA* and *Neurospora crassa NIT2*. Based on this study it is proposed that AreA is likely to contribute to the pathogenicity of *P. marneffei* by facilitating growth in the host environment and regulating the expression of potential virulence factors such as extracellular proteases.

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Introduction

The availability of nutrients, including an adequate nitrogen source, has a profound impact on the growth and development of many fungal species. Examples of the effects of nitrogen sources include the induction of pseudohyphal growth in *Saccharomyces cerevisiae*, haploid fruiting in *Cryptococcus neoformans* and filamentous growth of *Ustilago maydis* (Gimeno *et al.* 1992; Wickes *et al.* 1996; Smith *et al.* 2003). Many recent analyses have also linked the virulence of animal and plant pathogens to the ability to respond to nitrogen limiting conditions (Van den Ackerveken *et al.* 1994; Lau & Hamer 1996; Marzluf 1997). Ammonium and glutamine are preferentially utilised as nitrogen sources by fungi. In the absence of ammonium or glutamine various structural genes required for the uptake and catabolism of alternative nitrogen sources, including amino acids, amides, purines, nitrate, and nitrite are specifically activated. Multiple levels of control, involving global and pathway specific activation mechanisms, act to ensure that the appropriate structural genes are expressed to allow the catabolism of the nitrogen source available to the cell (reviewed in Marzluf 1997). In filamentous fungi, the global response to limiting nitrogen conditions is mediated by positively acting GATA-type zinc finger proteins, of which AreA from *Aspergillus nidulans* and NIT2 from *Neurospora crassa* are the most extensively characterised (Caddick *et al.* 1986; Stewart & Vollmer 1986; Fu & Marzluf 1987; Kudla *et al.* 1990).

The highly conserved DNA-binding domain present within the C-terminal portion of AreA/NIT2 contains a single zinc finger motif, represented by $C\text{-}X_2\text{-}C\text{-}X_{17}\text{-}C\text{-}X_2\text{-}C$ and recognises the core DNA sequence GATA, or more specifically HGATAR (Ravagnani *et al.* 1997). While large portions of the protein, particularly the N-terminal region, appear to be dispensable, the DNA-binding domain is essential for appropriate functioning of AreA/NIT2, with most mutations in this region resulting in loss-of-function phenotypes with strains unable to utilise nitrogen sources other than ammonium or glutamine. Aside from the DNA-binding domain, the extreme C-terminal

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residues are necessary for modulation of AreA/NIT2 function *via* interactions with the coactivator TamA and the nitrogen metabolite repressor protein NmrA (Xiao *et al.* 1995; Platt *et al.* 1996; Marzluf 1997; Andrianopoulos *et al.* 1998; Small *et al.* 1999).

Penicillium marneffei is a thermally dimorphic fungus capable of causing infection in immunocompromised individuals. This species displays filamentous hyphal growth at 25 °C and converts to a unicellular yeast dividing by fission upon transfer to 37 °C (Andrianopoulos 2002; Vanittanakom *et al.* 2006). The extent to which nutrient utilisation differs between 25 °C and 37 °C growth forms and the effects on the growth morphology are of particular interest. While the dimorphic switching program of *P. marneffei* is predominantly influenced by the incubation temperature, slight morphological variations have been observed during growth at 37 °C on nonpreferred nitrogen sources, with colonies displaying increased filamentation (Todd *et al.* 2003). The role of AreA/NIT2 related GATA factors in nitrogen regulation in filamentous fungi is likely to be conserved in *P. marneffei*, and may extend to the nutrient poor host environment. Therefore, the role of *P. marneffei* AreA in mediating growth and morphogenetic responses to nitrogen availability during both filamentous and yeast growth programs was investigated. Consistent with the role of other fungal GATA factors, AreA has been found to mediate the nitrogen response of *P. marneffei*. Interestingly, this occurs in both a nitrogen source and cell-type specific manner. The contribution of this highly conserved transcriptional regulator to the pathogenicity of *P. marneffei* is discussed.

Materials and methods

Molecular techniques

Plasmid DNA was isolated using the Wizard Plus SV DNA Purification System (Promega). Genomic DNA was prepared from frozen mycelia of *Penicillium marneffei* as described previously (Borneman *et al.* 2001). Southern blots were prepared with Hybond N+ membrane (Amersham) using standard procedures (Sambrook & Russell 2001). For screening of the *P. marneffei* genomic DNA lambda library, plaque lifts and the isolation of positive clones were performed according to the instructions of the λBlueSTAR vector system kit (Novagen). Hybridisations were performed with [α-^{32}P]-dATP-labelled DNA probes using standard methods (Sambrook & Russell 2001). DNA sequencing and analysis was performed according to Bugeja *et al.* 2010. Multiple sequence alignments were generated using ClustalW (Thompson *et al.* 2000) and BoxShade. A consensus phylogenetic tree was constructed from distance matrices of 1000 bootstrapped alignments using Phylip software package (Felsenstein 1989) and viewed using Treeview X (Page 1996).

Cloning and plasmid construction

A 2.5 kb *Bgl*II/*Stu*I fragment of pJAF4689 (Todd *et al.* 2005) containing *Aspergillus nidulans areA* was used as a heterologous probe to screen a *Penicillium marneffei* genomic DNA λ library at low stringency (40 % formamide, 2X SSC, 37 °C). The clone pHS5510 was found to contain similarity to the first 488aa of the 876aa *A. nidulans* AreA protein and was subsequently used as a homologous probe to rescreen the *P. marneffei* genomic DNA λ library at high stringency (50 % formamide, 0.1X SSC, 65 °C) to isolate pHS5575. The plasmid pHS6104 includes the entire *P. marneffei areA* gene and 1.2 kb and 0.7 kb of 5′ and 3′ noncoding sequence, respectively, generated from two subclones of pHS5575, a 2.7 kb *Sac*I/*Eco*RI fragment containing the 5′ end of *areA* and a 2.6 kb *Eco*RI fragment containing the 3′ end of *areA*, into pBluescript II SK$^+$.

In the gene deletion construct pHS6002, the *A. nidulans pyrG* blaster cassette from pAB4626 (Borneman *et al.* 2001) replaced a 349 bp region, between the *Sal*I and *Pst*I sites, which encodes the GATA-type zinc finger DNA-binding domain and is flanked by a total of 3.9 kb of genomic DNA specific to the *areA* locus of *P. marneffei*. This construct was excised as a linear 6.1 kb *Sac*II/*Xho*I fragment for transformation into *P. marneffei* strain SPM4.

Fungal strains and media

The *Penicillium marneffei* strains used in this study are listed in Table 1. The isolation and transformation of *P. marneffei* protoplasts were performed according to the method of Borneman *et al.* (2001). For selection of *pyrG*$^+$ transformants of strain SPM4, protoplasts were regenerated on osmotically stabilised protoplast medium (PM) containing 1.2 M sucrose and 10 mM ammonium tartrate $(NH_4)_2T$. Strains were grown on 1 % glucose minimal media (ANM) (Cove 1966) or yeast synthetic dextrose medium (SD) (Ausubel *et al.* 1994) with all nitrogen sources added at 10 mM final concentration as indicated. When required, medium was supplemented with 10 mM uracil to allow the growth of *pyrG*$^-$ strains. For the isolation of *pyrG*$^-$ revertants which had lost the *pyrG* blaster cassette,

Table 1 – *P. marneffei* strains used in this study.

Strain	Genotype	Source or reference
SPM3	*niaD1*	(Borneman *et al.* 2001)
SPM4	*niaD1*; *pyrG1*	(Borneman *et al.* 2001)
41.2.14	*niaD1*; *pyrG1*; *areA*$^{\Delta DBD}$::*AnpyrG*	This study
41.2.14-3	*niaD1*; *pyrG1*; *areA*$^{\Delta DBD}$	This study
48.2.1	*niaD1*; *pyrG1*; *areA*$^{\Delta DBD}$; [*areA* (pHS6104)]	This study
55.5.15	*niaD1*; *pyrG1*; *areA*$^{\Delta DBD}$; [*fmdS*::*lacZ* (pJAF4241)]; [*AnpyrG* (pAA4707)]	This study
61.2.3	*niaD1*; *pyrG1*; *areA*$^{\Delta DBD}$; [*fmdS*::*lacZ* (pJAF4241)]; [*AnpyrG* (pAA4707)]; [*areA* (pHS6104)]	This study
61.4.3	*niaD1*; *pyrG1*; *areA*$^{\Delta DBD}$; [*fmdS*::*lacZ* (pJAF4241)]; [*AnpyrG* (pAA4707)]; [*AnareA* (pKW5845)]	This study

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