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March 2007

## Functional characterization of the *Aspergillus fumigatus* calcineurin

Márcia Eliana da Silva Ferreira

*Faculdade de Ciências Farmacêuticas de Ribeirão, Universidade de São Paulo, Brazil*

Thorsten Heinekamp

*Department of Molecular and Applied Microbiology, Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knoell-Institute (HKI), Germany*

Albert Hartl

*Drug Testing Group, Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knoell-Institute (HKI), Germany*

Axel A. Brakhage

*Department of Molecular and Applied Microbiology, Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knoell-Institute (HKI), Germany*

Camile P. Semighini

*University of Nebraska-Lincoln*

*See next page for additional authors*

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**Authors**

Márcia Eliana da Silva Ferreira, Thorsten Heinekamp, Albert Hartl, Axel A. Brakhage, Camile P. Semighini, Steven D. Harris, Marcela Savoldi, Paula Fagundes de Gouvêa, Maria Helena de Souza Goldman, and Gustavo H. Goldman

## Functional characterization of the *Aspergillus fumigatus* calcineurin

Márcia Eliana da Silva Ferreira,<sup>a</sup> Thorsten Heinekamp,<sup>b</sup> Albert Härtl,<sup>c</sup> Axel A. Brakhage,<sup>b</sup> Camile P. Semighini,<sup>d</sup> Steven D. Harris,<sup>d</sup> Marcela Savoldi,<sup>a</sup> Paula Fagundes de Gouvêa,<sup>a</sup> Maria Helena de Souza Goldman,<sup>e</sup> and Gustavo H. Goldman<sup>a,\*</sup>

<sup>a</sup> Faculdade de Ciências Farmacêuticas de Ribeirão, Universidade de São Paulo, Brazil

<sup>b</sup> Department of Molecular and Applied Microbiology, Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knoell-Institute (HKI), Germany

<sup>c</sup> Drug Testing Group, Leibniz Institute for Natural Product Research and Infection Biology, Hans-Knoell-Institute (HKI), Germany

<sup>d</sup> Plant Science Initiative, University of Nebraska–Lincoln, USA

<sup>e</sup> Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, Brazil

\* Corresponding author. Fax: +55 16 633 1092; Email: [ggoldman@usp.br](mailto:ggoldman@usp.br) (G.H. Goldman).

**Abstract:** *Aspergillus fumigatus* is an aggressive opportunistic pathogen of humans as well as a major allergen. Environmental sensing and retrieving essential nutrients from the environment are general metabolic traits associated with the growth of this saprophytic fungus. Two important mediators of calcium signals in eukaryotic cells are the Ca<sup>2+</sup>-binding protein calmodulin and the Ca<sup>2+</sup>/calmodulin-dependent phosphatase calcineurin. Calcineurin is a heterodimer that consists of a catalytic subunit A and a Ca<sup>2+</sup>/calmodulin binding unit. We deleted the *A. fumigatus calA* gene, which encodes the calcineurin A catalytic subunit, and demonstrated that this gene is not essential in this fungus. The  $\Delta calA$  mutant strain has severe defects in growth extension, branching and conidial architecture. Furthermore, the *A. fumigatus*  $\Delta calA$  mutant strain has decreased fitness in a low dose murine infection and cannot grow in fetal bovine serum (FBS). After potassium phosphate was added to liquid FBS, the  $\Delta calA$  mutant strain could grow with the characteristic phenotype of the  $\Delta calA$  mutation. When *A. fumigatus* calcineurin is inhibited by tacrolimus in a phosphate depleted medium, there is a reduction in the inorganic phosphate transport and six putative phosphate transporter genes have altered mRNA levels. However, there is no effect on the acid phosphatase activity. These results suggest that calcineurin is involved in the regulation of the *PHO* pathway in *A. fumigatus*. Our work on calcineurin opens new venues for the research on sensing and nutrient acquisition in *A. fumigatus*.

**Keywords:** *Aspergillus fumigatus*, Calcineurin, Phosphate assimilation

### 1. Introduction

*Aspergillus fumigatus* is a primary and opportunistic pathogen as well as a major allergen (Denning, 1998, Casadevall and Pirofski, 1999 and Greenberger, 2002). Because *A. fumigatus* produces prodigious amounts of conidia, the human respiratory tract is continuously exposed to the fungus (Latge, 1999). This fungus is increasingly linked to severe asthma and sinusitis (Latge, 1999). *A. fumigatus* is isolated from human habitats and vegetable compost heaps (Beffa et al., 1998 and Latge, 1999). The incidence of invasive infection in im-

munocompromised individuals can be as high as 50% and the mortality rate is often ~50% (Denning, 1998 and Brakhage, 2005). In spite of its great medical and ecological importance, the basic biology of the organism is not very well understood. However, the recent completion of the *A. fumigatus* genome sequence (Nierman et al., 2005) has offered an opportunity to investigate complete pathways and classes of genes and to elucidate its main biological mechanisms.

Environmental sensing and the retrieval of essential nutrients from the environment are general metabolic traits that are associated with the growth of the saprophyte *A. fumigatus*

in inhospitable environments (Tekaiia and Latgé, 2005). Calcium is a ubiquitous second messenger that functions in signal transduction pathways in eukaryotic organisms. Calcium signaling is important for several cellular processes, including fertilization and development, exocytosis, muscle contraction, motility, chemotaxis, cell division, differentiation, programmed cell death and chromatin remodeling in multicellular eukaryotes (Carafoli, 2005). In filamentous fungi,  $\text{Ca}^{2+}$  is important for the regulation of hyphal morphogenesis, i.e., hyphal tip growth events involving branching and orientation (Jackson and Heath, 1993, Fox and Heitman, 2002 and Kraus and Heitman, 2003). Two important mediators of calcium signals in eukaryotic cells are the  $\text{Ca}^{2+}$ -binding protein calmodulin and the  $\text{Ca}^{2+}$ /calmodulin-dependent phosphatase calcineurin (Carafoli, 2005). Calcineurin is a serine/threonine-specific protein phosphatase heterodimer consisting of a catalytic subunit A and a  $\text{Ca}^{2+}$ /calmodulin binding unit. The association of the two subunits is essential for activity (Fox and Heitman, 2002). Calcineurin is required for signal transduction such as the activation of T cells (Schreiber and Crabtree, 1992). Calcineurin signaling is inhibited by the immunosuppressive drugs cyclosporin A (CsA) and FK506 (tacrolimus) that bind to the immunophilins cyclophilin and FKBP12, respectively (Liu et al., 1991). The cyclophilin-CsA and FKBP12-FK506 complexes then inhibit calcineurin (Liu et al., 1991). Recent investigations into the molecular mechanisms of pathogenesis in *C. albicans*, *C. neoformans*, and *A. fumigatus* have revealed an essential role for calcineurin in morphogenesis, virulence and antifungal drug action (Cruz et al., 2001a, Cruz et al., 2001b, Fox and Heitman, 2002, Kontonyannis et al., 2003, Steinbach et al., 2004a and Steinbach et al., 2004b). In *C. neoformans*, calcineurin is essential for growth at 37 °C but not at ambient temperature, and is also required for hyphal elongation during mating and haploid fruiting (Odom et al., 1997, Cruz et al., 2000, Cruz et al., 2001a and Fox et al., 2001). *C. albicans* calcineurin mutants had reduced virulence in a murine model of systemic infection (Cruz et al., 2002 and Sanglard et al., 2003). *C. albicans* calcineurin mutant strains failed to colonize and grow in kidneys of infected animals and were unable to survive when exposed to serum *in vitro* (Blankenship et al., 2003). The immunosuppressants CsA, FK506, and sirolimus (formerly called rapamycin) exhibit *in vitro* and *in vivo* activity against *C. albicans* and *C. neoformans*, including fungicidal synergy with azole antifungals (Cruz et al., 2001b). It has been reported that therapy with calcineurin pathway inhibitors and existing antifungal agents augment activity against *A. fumigatus* (Kontonyannis et al., 2003, Steinbach et al., 2004a and Steinbach et al., 2004b).

We have previously deleted the *cala* gene, which encodes the calcineurin A catalytic subunit CalA (Ferreira et al., 2006). The  $\Delta\text{cala}$  strain produced small sporulating colonies that showed comparable growth at different temperatures and increased branching when compared to the corresponding wild-type strain (Ferreira et al., 2006). Here, we extended our previous studies by showing that *A. fumigatus*

$\Delta\text{cala}$  mutant strain has severe defects in growth extension. Furthermore, the  $\Delta\text{cala}$  mutant strain has decreased fitness in a low dose murine infection and cannot grow in fetal bovine serum. Our data suggest a novel relationship between calcineurin function and inorganic phosphate acquisition in *A. fumigatus*.

## 2. Materials and methods

### 2.1. Strains and media methods

*Aspergillus fumigatus* strains used are CEA17 $\Delta\text{akuB}$ KU80 (wild-type),  $\Delta\text{cala}$ , and  $\Delta\text{cala calA}^+$ . Media were of two basic types. A complete medium with three variants: YAG (2% glucose, 0.5% yeast extract, 2% agar, trace elements), YUU (YAG supplemented with 1.2 g/l each of uracil and uridine) and liquid YG or YG + UU medium of the same compositions (but without agar). A modified minimal medium (MM: 1% glucose, original high nitrate salts, trace elements, 2% agar, pH 6.5); or minimal medium without  $\text{KH}_2\text{PO}_4$  (PF-MM). Trace elements, vitamins, and nitrate salts are described by Kafer (1977; Appendix, available on request from the author). Additionally, 10% fetal bovine serum (Gibco) was used as a medium. CsA, tacrolimus, and sirolimus are Neoral<sup>R</sup> Sandimmun (from Novartis), Prograf (from Janssen-Cilag), and Rapamune (from Wyeth), respectively.

For the disk diffusion assay, after standardized growth and harvest,  $1.0 \times 10^7$  *A. fumigatus* conidia were resuspended in 5 ml of YAG top-agar medium and poured onto YAG agar plates. Blank 6-mm-diameter paper disks (3 MM, Whatman) were impregnated with 25  $\mu\text{g}/\text{disk}$ , 100  $\mu\text{g}/\text{disk}$ , and 10  $\mu\text{g}/\text{disk}$  of tacrolimus, cyclosporin, and sirolimus, respectively, in YAG. The plates were incubated at 37 °C, and the diameters of the inhibition zones were evaluated after 24 or 48 h. Each disk diffusion assay was performed twice.

The viability of growing hyphae was measured as described by Bruschi et al. (2001). A suspension of conidiospores (approximately  $10^6$  conidia) was spread onto a MM plate using sterile glass spreader, and incubated for 16 h at 37 °C to produce a nonsporulating mycelial "mat." Mycelial "plugs" were cut from the mats using the wide end of a sterile Pasteur pipette. The plugs (four samples of each mutant) were transferred onto MM plates. The diameters of the resulting colonies were measured and radial growth was expressed in cm.

Phosphate transport in the wild-type and  $\Delta\text{cala}$  strains was measured by  $^{32}\text{P}$ -labelled orthophosphate. Freshly harvested conidia of wild-type strain was inoculated into 10 ml of liquid MM medium ( $1 \times 10^7$  conidia/ml) and grown for 16 h at 37 °C. Mycelia were aseptically transferred to 5 ml of liquid PF-MM free medium plus 1.0  $\mu\text{Ci}$   $^{32}\text{P}$ -labelled orthophosphate (Amersham Biosciences, UK) in the presence and absence of 2  $\mu\text{g}/\text{ml}$  of tacrolimus, and incubated at 37 °C for 2, 4, and 6 h. The mycelia were washed at least

four times with water, dried, weighted, and then phosphate incorporation was measured. The counts per minute (cpm) for triplicate samples were averaged. The same experimental design was repeated for wild-type and  $\Delta calA$  mutant strains phosphate incorporation.

### 2.2. Acid phosphatase enzymatic assay

Conidia ( $5 \times 10^7$ /ml) were inoculated in 250-ml Erlenmeyer flasks with 50 ml of MM and incubated at 37 °C/150 rpm for 16 h. Mycelia were filtered, washed with sterile water and transferred to 250-ml Erlenmeyer flasks with 50 ml of PF-MM. These cultures were subsequently incubated at 37 °C/150 rpm for 1, 2, and 4 h. One hundred microliters of each supernatant were added to a mixture reaction of 510  $\mu$ l (250  $\mu$ l sodium acetate buffer 100 mM pH 4.6, 10  $\mu$ l of  $MgCl_2$  100 mM, 250  $\mu$ l of *p*-nitro phenyl phosphate 10 mM), that was incubated at 37 °C for 60 min. After this period of time, the reaction was terminated by adding 1 ml of NaOH 1 M. The results were expressed as nanomole of *p*-nitrophenol liberated/mg of mycelial dry weigh/min (Caddick and Arst, 1986).

### 2.3. DNA manipulations and construction of the $\Delta calA$ strain

DNA manipulations were according to Sambrook et al. (1989). DNA fragment probes for Southern blots were labeled with [ $^{32}P$ - $\alpha$ ]dCTP using the Random Primers DNA labeling System kit (Invitrogen). PCR primers were designed for amplifying each DNA fragment necessary for PCR-mediated technique by using Primer Express Version 1.0 (Applied Biosystems) design software. For the construction of the deletion, the *A. fumigatus pyrG* gene was amplified from the plasmid pCDA21 (Chaverocche et al., 2000), and is referred as *zeopyrG* cassette because the amplified fragment also contains the zeocin-resistance gene. The PCR-mediated construction for the *calA* gene consisted of three initial amplifications that generated a 5'- and 3'-flanking region of *calA* gene and a final fusion PCR (for further details, see Ferreira et al., 2006). For the DNA fragments containing the flanking regions, genomic DNA was used as a template. The 5'-flanking fragment, which encompasses 2000 bp upstream of the ATG start codon was amplified with the primers calcineurin 1 (5'-ggttcattgggaccagacc-3') and calcineurin 2 (5'-ctgagaattcccttgcgcagtgtaactaag-3'). The 3' flanking region, which encompasses 2000-bp downstream of the stop codon of the *calA* genomic sequence, was amplified with the primers calcineurin 3 (5'-tgaggcgaattcaattgcttcggtcagctcca-3') and calcineurin 4 (5'-gcgaactctgaagctcgggt-3'). The *zeopyrG* cassette was amplified using plasmid pCDA21 as a template and the following primers: Calcin-ZeoF (5'-cacactgcgaaggaattctcagtcctctc-3') and Calcin-pyrG (5'-gaccgaagcaattgaattcgcctcaacaatgc-3). The final 6400-bp fusion PCR fragment was generated using the three previous DNA fragments as templates and calcineurin 1 and calcineurin 4 as primers. The 50  $\mu$ l amplification mixture included 1X platinum *Taq*

DNA Polymerase High Fidelity buffer (Invitrogen), 3 mM of  $MgSO_4$ , 20 pmol of each primer, 0.4 mM deoxynucleotide triphosphate (dNTP) mix, 1.0 U of *Taq* DNA HiFi platinum polymerase (Invitrogen), and 500 ng of genomic DNA or 100 ng plasmid. PCR amplification was carried out in a PTC100 96-well thermal cycler (MJ Research), at 94 °C for 2 min, and 30 times 94 °C for 1 min, 58–60 °C (depending on the fragment) for 1 min, and 68 °C for 7 min, followed by an extension step at 68 °C for 7 min. After the reaction, the PCR products were purified with a Qiagen PCR cleanup kit according to the manufacturer's instructions.

The  $\Delta calA$  strain was complemented by co-transformation of a linear fragment amplified from genomic DNA by using primers calcineurin 1 and 4, and a derivative of plasmid pAN7-1 (pHAT $\alpha$ ; Herrera-Estrella et al., 1990), that contains the *hph* gene encoding the *Escherichia coli* hygromycin phosphotransferase and confers resistance to hygromycin. Transformation of *A. fumigatus* strain  $\Delta calA$  was according to the procedure of Osmani et al. (1987) using 5  $\mu$ g of linear or circular DNA fragments. Transformants were scored for their ability to grow on YG medium or in YUU plus 1 M sucrose, in the presence of 200  $\mu$ g/ml of hygromycin (Sigma–Aldrich). Southern analysis demonstrated that the deletion cassette had integrated at the *calA* locus. The  $\Delta calA$  *calA*<sup>+</sup> strain ( $\Delta calA$  complemented with the *calA* gene) shows essentially the same behavior of the wild-type strain.

### 2.4. Real-time PCRs

All the reactions were performed using an ABI Prism Sequence Detection System (Perkin-Elmer Applied Biosystem, USA). The SYBR<sup>®</sup> Green PCR Master Mix was used for PCRs. The thermal cycling conditions comprised an initial step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s. The reactions and calculations were performed according to Semighini et al. (2002). The oligonucleotides used are described in Table 1.

### 2.5. Virulence assays

Virulence of different *A. fumigatus* strains was determined in a murine low dose model for invasive aspergillosis as described previously (Liebmann et al., 2004 and Ferreira et al., 2006). Survival of female BALB/c mice (Harlan Winkelmann, Borchon, Germany) infected intranasally with conidia of *A. fumigatus* wild-type,  $\Delta calA$  *calA*<sup>+</sup>, and  $\Delta calA$  strains were monitored. Mice were immunosuppressed by intraperitoneal injection of cyclophosphamide (150 mg/kg body weight) every third day, starting on day-4 prior to infection. Additionally, a single dose of cortisone acetate (200 mg/kg body weight) was injected subcutaneously on day-1. On day 0, mice were infected with  $3 \times 10^4$  of viable freshly harvested *A. fumigatus* conidia. Animals were tested in cohorts of five animals in sterile aerated cages. Survival was monitored at least twice a day for a time period of 14 days and moribund animals were sacrificed.



**Table 1.** List of primers used in this work for the real-time RT-PCRs

Primer	Sequences <sup>a</sup>	Gene
634FL/663RU	5'-GTCGTACAGAGCCTCGTTGTCG-3'	Afu1g10910
634FL	5'-CGAGCCCTCTCCGTTACACCAGCT[FAM]G-3'	
MS1-F	5'-TGTTTCGTGCCTATATGAC-3'	Afu4g03610
MS1-R	5'-GCTTCTGGAGGACGGACTGG-3'	
MS2-F	5'-CCTCCATCTACCTCACCGT-3'	Afu3g03010
MS2-R	5'-GAGTGGTTCGAGACCGGCATAC-3'	
MS3-F	5'-CGGGCCGTGAATGCCTATGT-3'	Afu1g04290
MS3-R	5'-TGGATGTACGACTTGAGCC-3'	
MS4-F	5'-TGAAGAGCGCACTGGAGT-3'	Afu5g01960
MS4-R	5'-GGCTTCCCGAACACATGGAT-3'	
MS5-F	5'-TGTTGGCCTTATGTGTTTC-3'	Afu6g07750
MS5-R	5'-CACGTCATTGGGAGCGTTGT-3'	
MS6-F	5'-GCCTTCGATGCTCTCATTCG-3'	Afu2g10690
MS6-R	5'-CTGCCCAGTAGACACCATC-3'	
MS7-F	5'-GTGGAGACTGGTGGTGGGAGTT-3'	Afu4g09210
MS7-R	5'-AGCACTGAGGGGATAATC-3'	
MS8-F	5'-GGATCACGGCGTCAAGTGGT-3'	Afu7g06350
MS8-R	5'-GCTTGAACAGCAGAGGAC-3'	
MS9-F	5'-GCTGGCGCGGTAGATACTGA-3'	Afu8g01850
MS9-R	5'-AATCACAAGGAACCACAC-3'	

<sup>a</sup> 6FAM, 6-carboxyfluorescein.

## 2.6. Microscopy

Conidiospores from wild-type,  $\Delta calA$   $calA^+$ , and  $\Delta calA$  strains mutant were germinated at 37 °C on glass coverslips arrayed on the bottom of a plastic 100 mm Petri dish containing the appropriate growth medium. Coverslips with adherent hyphae were fixed and stained as previously described (Harris et al., 1994). Nuclei were visualized by staining with Hoechst 33258 or DAPI, and septa were stained using Calcofluor. After staining, coverslips were washed in sterile water and mounted in *n*-propyl gallate. Slides were viewed using an Olympus BX51 fluorescent microscope. Images were captured with a Photometrics CoolSnap HQ CCD camera (Roper Scientific), and processed using IPLab software (Scanalytics, Inc.) and Adobe PhotoShop 6.0. Confocal images were obtained with an Olympus FW500/BX61 confocal laser scanning microscope using the following laserlines: 405nm for Hoechst 33,258 and 488 nm for FITC. Images were captured by direct acquisition with a Z step of 1  $\mu$ m. They were subsequently processed using Adobe PhotoShop 6.0.

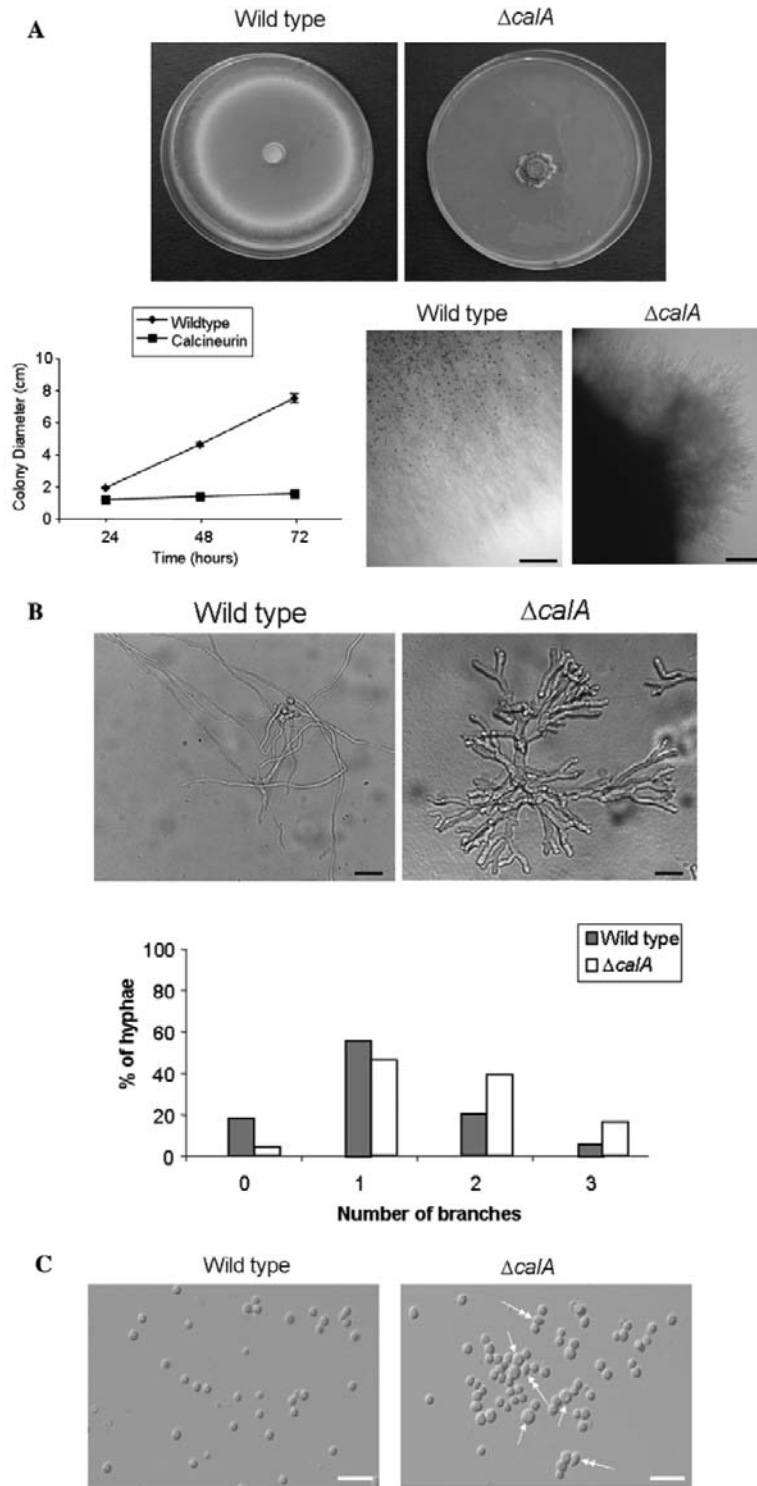
For scanning electron microscopical analysis of conidia, strains were cultivated for three days on MM agar plates and conidia were sampled directly on double-sided adhesive tape. Microscopy was performed on a scanning electron microscope LEO-1450VP (Zeiss, Germany).

## 3. Results

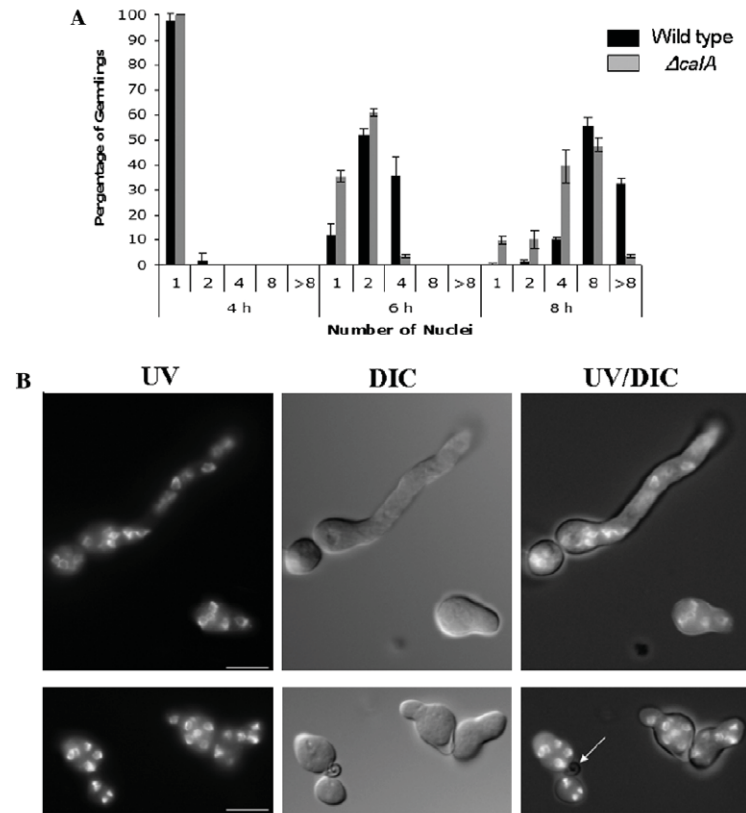
### 3.1. *Aspergillus fumigatus* calcineurin catalytic subunit inactivated mutant ( $\Delta calA$ ) has severe defects in growth extension

We had previously deleted the *calA* gene that encodes the calcineurin A catalytic subunit ( $\Delta calA$  strain; Ferreira et al., 2006). As observed before, primary transformants displayed

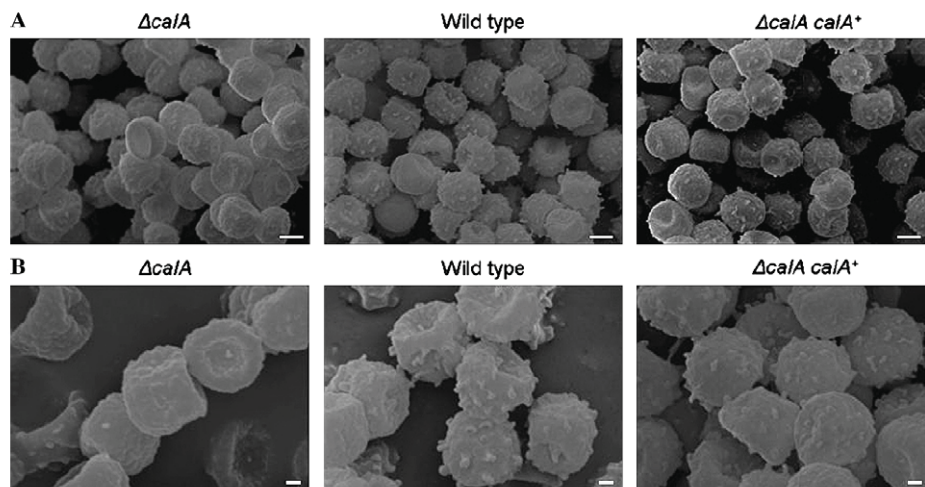
a pronounced phenotypic defect, i.e., small sporulating colonies (Figure 1A, upper panel; Ferreira et al., 2006). The diameter of the wild-type and  $\Delta calA$  colonies was measured daily after the transfer of mycelial plugs which all had about one cm of diameter (Figure 1A, lower left panel). Wild-type colonies radiate outwards and occupy the entire plate surface by 3 days (average diameter of 7.5 cm). The  $\Delta calA$  strain has undulating colonies that display limited growth (average diameter of 1.5 cm). The wild-type strain presented the expected hyphal margin at the edge of the colony (Figure 1A, lower right panel). Three independent measurements revealed that these hyphae extended 6.3 cm (standard deviation,  $SD = 1.53$ ) after three days. By contrast, the hyphal margin of the  $\Delta calA$  strain did not extend normally, resulting in a compact colony (Figure 1A, lower right panel). The morphology of the  $\Delta calA$  germlings showed increased apical branching when compared to the corresponding wild-type strain (Figure 1B). Measurements of the number of branches show that 60% of wild-type hyphae possess at least one branch and 30% have two or more, compared to 45 and 50% of  $\Delta calA$  hyphae in each of these respective categories (Figure 1B, lower panel). The  $\Delta calA$  strain possessed normal microtubules; however, although the conidiophores are normal, only having smaller vesicles when compared to the wild-type (data not shown), the calcineurin mutation influenced conidial morphology (Figure 1, Figure 2 and Figure 3). Wild-type spores presented a uniform morphology and size, whereas 47.5% ( $n = 200$ ,  $SD = 7.8$ , two independent experiments) of  $\Delta calA$  spores exhibited abnormal size (represented by arrows in Figure 1C) or “tear-shape” morphology (represented by double arrows in Figure 1C). These abnormal spores have no nucleus and do not germinate; however this is not due to any technical problems since wild-type resting conidia stained with Hoescht (Figure 1 and Figure 2B).



**Figure 1.** Calcineurin is required for colony extension: spores from each strain were inoculated in MM plates (plus supplements) and incubated at 37 °C overnight to produce a nonsporulating mycelial “mat.” Mycelial “plugs” were cut from the mats using the wide end of a sterile Pasteur pipette. The plugs were transferred onto MM plates and incubated for 3 days at 37 °C. (A) Colony morphology (upper panels) and growth rate of wild-type (diamond shape) and  $\Delta calA$  (square shape) colonies (lower left panel). The diameter of colonies was measured daily after the transfer of the mycelial plugs which were originally 1 cm in diameter. The average of three independent experiments is shown. Lower right panels are higher magnification images showing the edge of the colonies represented in the plates of the upper panels. Wild-type, bar, 100  $\mu$ m;  $\Delta calA$ , bar, 10  $\mu$ m. (B) Calcineurin is required for proper hyphal branching. Conidia of wild-type and  $\Delta calA$  mutant strains were inoculated in liquid YG and incubated at 37 °C for 10 h (upper panels; wild-type, bar, 27  $\mu$ m;  $\Delta calA$ , bar, 22  $\mu$ m). Samples were fixed and stained with DAPI and calcofluor, then analyzed by light microscopy and the number of branches counted. (C) Calcineurin influences spore morphology. Wild-type spores display uniform morphology and size, whereas  $\Delta calA$  spores (B) exhibit abnormal size (represented by arrows) or “tear-shape” morphology (represented by double arrows). Bars, 5  $\mu$ m. Those abnormal spores have no nucleus.



**Figure 2.** (A) Wild-type and mutant conidia were germinated on coverslips in proper liquid media for 4, 6, and 8 h at 37 °C. Coverslips were fixed and stained with Hoechst 33258. Nuclei were visualized with fluorescence microscopy and the number of nuclei per germling was analyzed. For each sample, 200 germlings were examined in three independent experiments. (B) The wild-type and  $\Delta calA$  mutant (lower panel) conidia germination after 8 h. Note that the defective spore with tear shape (arrow) lacks nuclear staining, appears lysed and does not germinate. Bars, 10  $\mu$ m (UV and DIC are UV light and visible light fields, respectively).

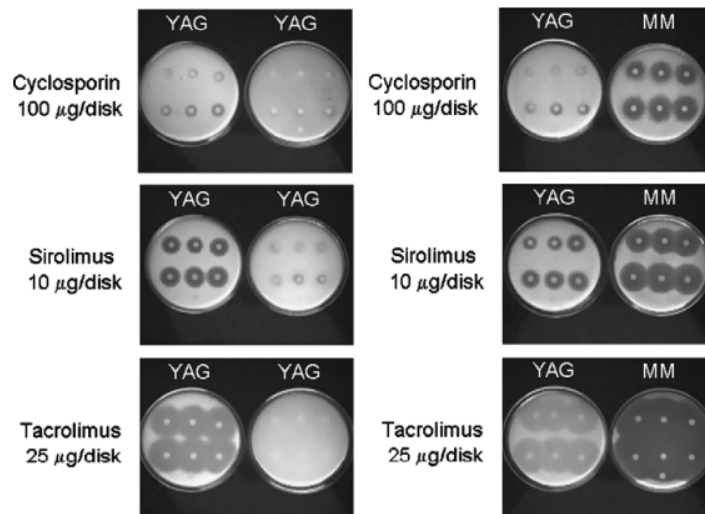


**Figure 3.** Conidia of the  $\Delta calA$  mutant strain have reduced amounts of rodlets. Strains were cultivated for three days on MM agar plates and conidia were sampled directly on double-sided adhesive tape. Microscopy was performed on a scanning electron microscope LEO-1450VP (Zeiss, Germany). (A) 8000 times increase (bar, 300 nm); (B) 16,000 times increase (bar, 1.0  $\mu$ m).

The wild-type and  $\Delta calA$  mutant strains display comparable nuclear division kinetics up to 4 h germination (Figure 2A). However, there is enhanced nuclear division kinetics in the

wild-type strain with about 35 and 32% germlings having four and more than eight nuclei per germling at 6 and 8 h germination, respectively (Figure 2A). In contrast, the  $\Delta calA$  mutant





**Figure 4.** The  $\Delta calA$  mutant strain shows resistance to immunosuppressants and the wild-type drug-sensitivity is increased in minimal medium. For disk diffusion assay, after standardized growth and harvest,  $1.0 \times 10^7$  *A. fumigatus* conidia were resuspended in 5 ml of YAG or MM top-agar medium and poured onto YAG or MM agar plates. Blank 6-mm-diameter paper disks (3MM, Whatman) were impregnated with 25  $\mu\text{g}/\text{disk}$ , 100  $\mu\text{g}/\text{disk}$ , and 10  $\mu\text{g}/\text{disk}$  of tacrolimus, cyclosporin, and sirolimus, respectively. After drying, they were placed onto inoculated agar plates. The plates were incubated at 37 °C, and the diameters of the inhibition zones were evaluated after 24 h. Each disk diffusion assay was performed twice and each plate has six disks repetitions of the same concentration of the specific immunosuppressor and a seventh disk containing the same volume of dimethyl sulfoxide that was used to dilute the immunosuppressor and was used as a negative control for the experiment. (A) The wild-type and  $\Delta calA$  strains were inoculated in YAG medium plus different immunosuppressors. (B) The wild-type strain was inoculated in YAG and MM media plus different immunosuppressors.

strain has about 4 and 3% germlings having four and more than eight nuclei per germling at 6 and 8 h germination, respectively (Figure 2A). We have not observed any effect of different temperatures, pH values, osmotic conditions, carbon sources, and increasing concentrations of Triton X-100,  $\text{CaCl}_2$ , and  $\text{MnCl}$  on the growth of the  $\Delta calA$  mutant strain (data not shown). These results suggest that the *calA* gene is not essential in *A. fumigatus*, but its deletion confers a severe growth defect and decreased rate of nuclear division kinetics.

Scanning electron microscopy revealed a morphological conidial defect in the  $\Delta calA$  mutant (Figure 3). The conidia of wild-type *A. fumigatus* are coated with hydrophobic proteins called rodlets which extend out from the conidial surface (Thau et al., 1994 and Girardin et al., 1999). The  $\Delta calA$  mutant conidia seemed not to have these surface rodlets and instead contain smooth conidial surfaces (Figure 3).

Immunosuppressors such as tacrolimus (FK506) and CsA mediate their immunosuppressive effects with the peptidyl-prolyl isomerases, FKBP12 and cyclophilin A, respectively (Liu et al., 1991). Calcineurin is the target of the inhibitory FKBP12-FK506 and cyclophilin A-CsA complexes. CsA, tacrolimus, and sirolimus exhibit *in vitro* and *in vivo* activity against *A. fumigatus* (Steinbach et al., 2004a and Steinbach et al., 2004b). Thus, we tested the effect of the immunosuppressants on  $\Delta calA$  growth. As previously reported the *A. fumigatus* wild-type strain is sensitive to CsA, tacrolimus, and sirolimus (Kontonyannis et al., 2003, Steinbach et al., 2004a and Steinbach et al., 2004b; Figure 4A). However, the  $\Delta calA$  strain displays almost no sensitivity to CsA and sirolimus, and no sensitivity at all to tacrolimus (Figure 4A). These results suggest that the  $\Delta calA$  mutation confers increased resistance

to immunosuppressors. We also decided to evaluate if the sensitivity of the wild-type strain to immunosuppressors could be modulated by the nutrient conditions. Thus, we grew the wild-type strain in YAG or MM in the presence of the immunosuppressants. As shown in Figure 4B, sensitivity to the immunosuppressants increased more than 50% when the wild-type strain was grown in MM. These results strongly indicate that calcineurin is important for growth in low nutrient conditions.

### 3.2. *Aspergillus fumigatus* $\Delta calA$ mutant strain cannot grow in fetal bovine serum

To determine a possible influence resulting from deletion of the *calA* gene, the corresponding deletion mutant was tested in an animal model. Virulence of *A. fumigatus* strain  $\Delta calA$  in comparison to strains  $\Delta calA calA^+$ , and wild-type, was determined in a murine low dose model for invasive aspergillosis as optimized by Liebmann et al. (2004). Considering the decreased viability of  $\Delta calA$  conidia, mice were exposed to the same number of viable conidia for each strain ( $3 \times 10^4$  viable conidia). Infection with either the complemented  $\Delta calA$  ( $\Delta calA calA^+$ ) or the wild type strains resulted in a mortality rate of 70–90% after 10 days post inoculation of the conidia while the *calA* deletion strain resulted in a mortality rate of 20% (data not shown). These results indicate that the *calA* mutation is causing loss of fitness when the  $\Delta calA$  strain is grown in the animal model when compared to the corresponding complemented and wild-type strains.

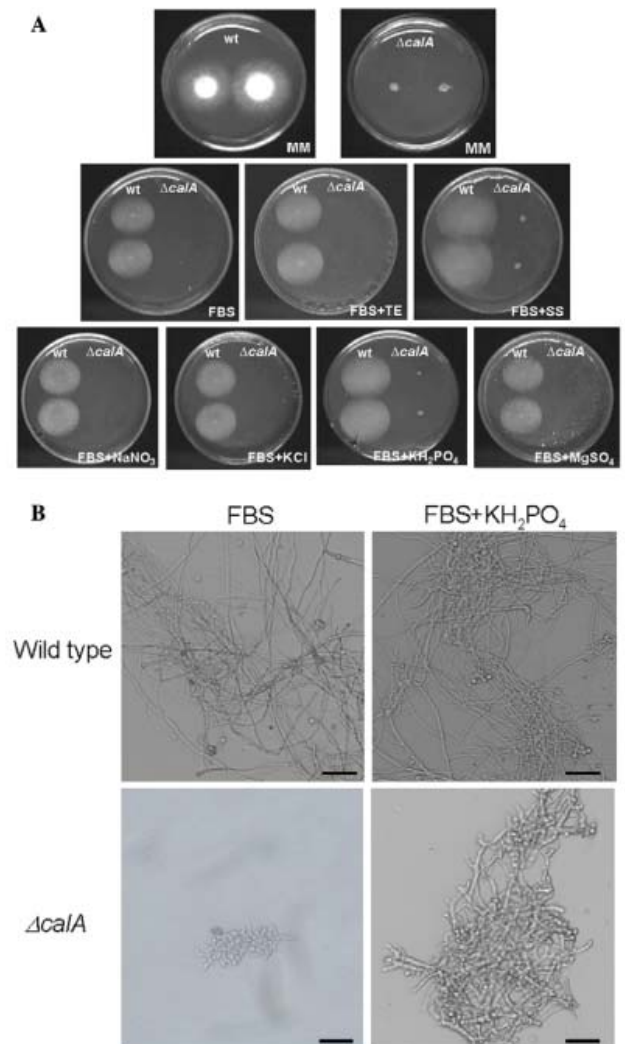
Next step, we tested the  $\Delta calA$  mutant strain for growth in 10% FBS. Although the wild-type strain can grow in 10% FBS, the  $\Delta calA$  strain cannot (Figure 5A). Since the  $\Delta calA$

strain can grow in MM to the same extent as in YAG medium (data not shown), we hypothesize that a component of the MM would be able to complement the FBS growth defect from the  $\Delta calA$  strain. The MM is composed of glucose, trace elements, and salt solution which were individually added to 10% FBS medium, and only the salt solution had obvious effects on the growth of the calcineurin mutant in 10% FBS (Figure 5A). The salt solution is composed of sodium nitrate, potassium chloride, potassium phosphate, and magnesium sulphate; only 11 mM potassium phosphate could partially suppress the growth defect of the  $\Delta calA$  mutant strain (Figure 5A). Even the wild-type can grow faster when potassium phosphate was added to 10% FBS medium (Figure 5A). When potassium phosphate was added to liquid 10% FBS, the  $\Delta calA$  mutant strain grew and displayed the characteristic phenotype of the  $\Delta calA$  mutation (Figure 5B). The  $\Delta calA calA^+$  strain showed the same behavior of the wild-type strain (data not shown). Since only potassium phosphate, but not potassium chloride, could suppress the absence of growth of the  $\Delta calA$  mutant strain, we conclude that the *calA* gene is essential for the utilization of inorganic phosphate ( $P_i$ ).

In *Saccharomyces cerevisiae*, as a response to  $P_i$  limitation, at least 22 *PHO*-related genes are derepressed, leading to elevated transcript levels, as judged by DNA microarray analysis together with genetic and biochemical studies (for a review, see Persson et al., 2003). To monitor the status of the *PHO* pathway, the activity of acid phosphatase which is transcriptionally induced as a response to  $P_i$  starvation is commonly used (Persson et al., 2003). Thus, we decided to verify if when the calcineurin function is restrained, *A. fumigatus* is still able to secrete acid phosphatase. First, wild-type strain was grown for 16 h in liquid MM and then mycelia were transferred to liquid PF-MM either in the presence or absence of tacrolimus, and acid phosphatase activity was measured after 1, 2, and 4 h (Table 2). *A. fumigatus* mycelia grown either in the absence or presence of tacrolimus displayed comparable levels of acid phosphatase activity (Table 2). These results strongly suggest that the *A. fumigatus PHO* pathway is still active when the calcineurin is inhibited in phosphate-depleted medium.

The absence of growth in the FBS medium could be related to a decreased transport of inorganic phosphate into *A. fumigatus* cells. To investigate this hypothesis, we have grown the wild-type in liquid MM, the mycelia were transferred to liquid PF-MM either in the presence or absence of a sub-inhibitory concentration of tacrolimus (2  $\mu$ g/ml) plus inorganic  $^{32}P$ , grown for 2, 4, and 6 h at 37 °C, and then the phosphate incorporation was measured. There is a reduction of about 22% in the phosphate incorporation when calcineurin is inhibited (Table 3). We also measured the phosphate incorporation in the  $\Delta calA$  mutant strain. There is a reduction of about 15% (2 h) to 35% (4 and 6 h) in the phosphate incorporation into the  $\Delta calA$  mutant strain (Table 3).

At low external  $P_i$  concentrations, the expression of genes encoding high-affinity transporter encoding genes such as *S. cerevisiae PHO84* is up-regulated; this occurs by binding of the



**Figure 5.** *Aspergillus fumigatus*  $\Delta calA$  mutant cannot grow in fetal bovine serum without supplemented phosphate. (A) The wild-type and  $\Delta calA$  mutant strains were point inoculated either in MM and grown for 48 h at 37 °C (first row), or 10% FBS, 10% FBS + TE (trace elements according to Kafer, 1977), and 10% FBS + SS (salts solution according to Kafer, 1977) (second row), or 10% FBS +  $NaNO_3$ , 10% FBS + KCL, 10% FBS +  $KH_2PO_4$ , 10% FBS +  $MgSO_4$  (third row) and grown for 72 h at 37 °C. (B) Conidia from the wild-type and  $\Delta calA$  mutant strains were inoculated in 10% FBS and FBS +  $KH_2PO_4$  solid media and grown for 48 h at 37 °C. Bar, 33  $\mu$ m. In both experiments in solid and liquid medium, the plates and tubes were incubated up to 10 days at 37 °C and no growth was observed in the  $\Delta calA$  mutant.

**Table 2.** Calcineurin inhibition does not affect the acid phosphatase activity<sup>a, b</sup>

	1 h	2 h	4 h
PF-MM	305.0 $\pm$ 49.5	338.0 $\pm$ 69.7	478.6 $\pm$ 50.6
PF-MM + Tacro	313.5 $\pm$ 50.5	442.6 $\pm$ 104.4	313.5 $\pm$ 50.5

<sup>a</sup> Results are the average of three repetitions.

<sup>b</sup> Acid phosphatase activity of culture filtrates expressed as nmol of *p*-nitrophenol liberated/mg of mycelial dry weight/min.

**Table 3.** Inhibition of the calcineurin decreases the inorganic phosphate transport in *A. fumigatus*

	2 h <sup>a</sup>	4 h	6 h
Wild-type PF-MM	5.55 ± 0.95	28.04 ± 3.12	36.48 ± 2.98
Wild-type PF-MM +TACRO	4.41 ± 0.25	21.90 ± 3.83	28.55 ± 1.59
$\Delta calA$ PF-MM	4.73 ± 0.80	18.71 ± 5.03	23.27 ± 6.16

<sup>a</sup> Activity is expressed as  $\times 10^3$  cpm/mg dry weight and are the average of three repetitions  $\pm$  standard deviation.

**Table 4.** Expression of putative transporter encoding genes when *A. fumigatus* is grown in the presence of tacrolimus

Transporter genes <sup>a</sup>	0 h <sup>b</sup>	8 h	8 h + TACRO	Changes (fold number)
Afu4g03610	1.0	412	19	Decrease 22 X
Afu3g03010	1.0	1,081	981	No change
Afu1g04290	1.0	1.60	2.75	Increase 1.7 X
Afu5g01960	1.0	1.79	2.74	Increase 1.53 X
Afu6g07750	1.0	5.25	1.60	Decrease 3.30 X
Afu2g10690	1.0	6.0	10.0	Increase 1.7 X
Afu4g09210	1.0	10.6	13.0	No change
Afu7g06350	1.0	259.6	217.5	No change
Afu8g01850	1.0	8.5	34.7	Increase 4.1 X

<sup>a</sup> The strains were grown for 16 h in MM and then transferred to PF-MM for 8 h, in the presence and absence of 2  $\mu$ g/ml of tacrolimus (TACRO) both at 37 °C.

<sup>b</sup> The measured quantity of the mRNA from the transporter genes in each of the treated samples was normalized using the  $C_t$  values obtained for the beta tubulin RNA amplifications run in the same plate. The relative quantitation of transporter and tubulin gene expression was determined by a standard curve (i.e.,  $C_t$  values plotted against logarithm of the DNA copy number). Results of four sets of experiments were combined for each determination. The values represent the number of times the genes are expressed compared to the time zero control (represented absolutely as 1.00).

active transcription factors Pho4p and Pho2p to the promoter regions of the *PHO* genes (Persson et al., 2003). The *PHO* pathway is poorly characterized in *A. fumigatus*, however we were able to identify nine genes encoding putative proteins that show high similarity to *S. cerevisiae* phosphate transporters (Blastp e-values < 1e-57;  $\geq$  40% identity and  $\geq$  50% similarity; Afu4g03610, Afu3g03010, Afu1g04290, Afu6g07750, Afu4g09210, Afu2g10690, Afu7g06350, Afu8g01850, and Afu5g01960). Again, we used the same experimental strategy by inhibiting calcineurin function through the use of tacrolimus, and the expression of all these nine transporter encoding genes was assessed by using real-time RTPCR (Table 4). There are two genes that showed decreased mRNA accumulation when *A. fumigatus* was exposed to tacrolimus (Afu4g03610 and Afu6g07750). Interestingly, there are four genes that showed increased mRNA accumulation when calcineurin was inhibited (Afu1g04290, Afu5g01960, Afu2g10690, and Afu8g01850). The mRNA accumulation of three genes (Afu3g03010, Afu4g09210, and Afu7g06350) was not significantly affected by growth in the presence of tacrolimus (Table 4).

Taken together these results suggest that there is a reduction in inorganic phosphate transport and mRNA accumula-

tion of six putative phosphate transporter encoding genes is altered when calcineurin is inhibited in phosphate-depleted medium.

#### 4. Discussion

*Aspergillus fumigatus* is a saprophytic fungus that is important for nutrient recycling in nature. However, *A. fumigatus* can also cause human disease as an opportunistic pathogen, and depending on the immune status of the patient, this fungus can elicit responses ranging from allergy to disseminated and frequently fatal infection. Sensing and nutrient acquisition mechanisms are essential for the fungus in order to discriminate in which environment it is growing. *A. fumigatus* senses and responds to its immediate environment via signal transduction cascades, which transduce information from the outside environment to the inside the cell. One of these mechanisms of intracellular signaling includes  $Ca^{2+}$  ions, which bind to and activate calmodulin. The  $Ca^{2+}$ -calmodulin complex then binds to and activates calmodulin-dependent enzymes, such as the protein phosphatase calcineurin. Here, we show that calcineurin is not an essential gene in *A. fumigatus*, and present the roles of calcineurin in regulating differentiation and fitness in this opportunistic pathogen. In agreement to our results, Steinbach et al. (2006) have also recently shown that a mutant of *A. fumigatus* lacking the calcineurin A (*cnaA*) catalytic subunit exhibited defective hyphal morphology related to apical extension and polarized growth, which resulted in drastically decreased filamentation. Lung tissue from animals infected with the delta *cnaA* mutant showed a complete absence of hyphae, in contrast to tissue from animals infected with the wild-type and complemented strains.

##### 4.1. Calcineurin is essential for *A. fumigatus* colony extension

Calcineurin plays an important role in the control of cell morphology and virulence in fungi. Growing hyphae contain a tip-high  $Ca^{2+}$  gradient thought to be crucial for establishing and maintaining apical organization, morphogenesis, and growth (Jackson and Heath, 1993). In *N. crassa*, the function of calcineurin was investigated by induction of antisense RNA expression and reduced levels of *can-1* (encoding the catalytic subunit) mRNA and decreased calcineurin enzyme activity (Prokisch et al., 1997). Induction of the antisense RNA expression, caused growth arrest preceded by an increase in hyphal branching, changes in hyphal morphology and concomitant loss of the distinctive tip-high  $Ca^{2+}$  gradient typical for growing wild-type hyphae. In the same organism, the gene encoding the regulatory subunit (*cnb-1*) was inactivated by an insertional mutagenesis procedure followed by a screening protocol designed to isolate mutants affected in the ability to regulate entry into conidiation (Kothe and Free, 1998). When *cnb-1* was inactivated through RIP, the mutant showed a more severely restricted colonial growth pattern that consisted en-



tirely of highly branched hyphae of swollen septated cells. It has been reported that calcineurin (*cnaA*) is an essential gene in *A. nidulans*, (Rasmussen et al., 1994). However, in contrast to these authors we have observed that the *cnaA* gene is not an essential gene and that the deletion strain displays a very closely related morphological phenotype to that seen for *A. fumigatus* (Fagundes, unpublished results).

In *C. neoformans*, calcineurin is required for growth at 37 °C, virulence, and mating (Odom et al., 1997, Fox et al., 2001 and Cruz et al., 2001a). Calcineurin is not essential for the initial fusion event, but is required for hyphal elongation and survival of the heterokaryon produced by cell fusion (Cruz et al., 2001a). It is also required for hyphal elongation in diploid strains and during asexual haploid fruiting of MAT $\alpha$  cells in response to nitrogen limitation (Cruz et al., 2001a). Additionally, calcineurin regulates filamentation and 37 °C growth via distinct mechanism. In *C. albicans*, deletion of the gene encoding the calcineurin A subunit (*CNA1*) affected colony morphology in several media known to modulate the *C. albicans* dimorphic switch (Sanglard et al., 2003).

We found that calcineurin is required for *A. fumigatus* colony extension and for normal conidial morphology. The absence of calcineurin also showed decreased nuclear division kinetics and increased branching in *A. fumigatus*. The  $\Delta$ *cala* mutant conidia displayed abnormal morphology and are frequently anucleate. Analysis by scanning electronic microscopy showed that the surface of the conidia harvested from the  $\Delta$ *cala* mutant have disorganized prominent protrusions. The interwoven proteinaceous microfibrils that decorate the outermost cell wall layer of *Aspergillus* conidia are called rodlets (Thau et al., 1994 and Girardin et al., 1999). Rodlets are composed of hydrophobins that confer physicochemical properties to mediate conidial dispersal, and possibly, cellular interactions (Thau et al., 1994 and Girardin et al., 1999). It is possible that the  $\Delta$ *cala* mutant has decreased expression of hydrophobins and consequently decreased host tissue adhesion. Preliminary analysis in our laboratory indicates that *A. fumigatus*  $\Delta$ *cala* mutant has decreased adhesion to laminin (data not shown).

#### 4.2. Calcineurin deletion mutant cannot grow in FBS

We have shown that the *A. fumigatus* calcineurin deletion mutant has decreased virulence in a murine low dose model. Calcineurin is also important for virulence in *C. albicans* and *C. neoformans* (Cruz et al., 2001b, Bader et al., 2003 and Sanglard et al., 2003). However, the observed decreased virulence in the  $\Delta$ *cala* mutant is most probably due to the decreased fitness of this strain. Furthermore, calcineurin is essential for *A. fumigatus* growth in bovine fetal serum, but growth can be rescued if inorganic phosphate is added to the serum, indicating that calcineurin is involved in phosphate acquisition in 10% FBS. It remains to be investigated if the FBS contains molecules that can chelate the free phosphate or inhibitors affecting phosphate uptake.

Phosphate is an ion that is essential for fungal growth and the amount of phosphate present in the serum is below 1 mM (Tekaiia and Latgé, 2005). Actually, the  $\Delta$ *cala* mutant strain is also not able to grow in MM supplemented with inorganic phosphate in concentrations  $\leq$  1 mM (data not shown). Cellular acquisition, storage and release, and metabolic integration of inorganic phosphate (Pi) rely on the essential participation of numerous enzymes, such as extracellular acid phosphatases, phosphodiesterases, phosphate transporters, polyphosphate kinases, alkaline phosphatases, and endopolyphosphates (for a review, see Persson et al., 2003). In *S. cerevisiae*, the activities of the enzymes needed for intracellular Pi homeostasis are subjected to regulation via the Pi signal transduction pathway (*PHO* pathway) (Persson et al., 2003). In *S. cerevisiae*, a complex of cyclin-dependent kinase (CDK), Pho85p, one of its cyclins, Pho80p, and the CDK inhibitor (CKI), Pho81p, together with the transcription factors Pho4p and Pho2p constitute the core components of the transcriptional regulation of *PHO* genes (Persson et al., 2003).

To monitor the status of the *PHO* pathway, the activity of secreted acid phosphatase (the gene product of *PHO5*, which is transcriptionally induced as a response to P<sub>i</sub> starvation) is commonly used (Persson et al., 2003). Thus, we investigated the activity levels of acid phosphatase when *A. fumigatus* wild-type is submitted or not to calcineurin inhibition by tacrolimus in PF-MM. Calcineurin inhibition does not affect the acid phosphatase activity, suggesting that the *PHO* pathway is also active in the  $\Delta$ *cala* mutant strain.

The systems for Pi transport have been characterized as a low-affinity (that assures a supply of Pi at normal or high external Pi concentrations) and a high-affinity (activated in response to Pi starvation) process (Persson et al., 1999). We have shown that there is a reduction in the inorganic phosphate transport when *A. fumigatus* calcineurin is inhibited. In *S. cerevisiae*, the genes are also regulated at the transcriptional level and we were able to show that the mRNA accumulation of some *A. fumigatus* putative phosphate transporter genes was altered when calcineurin is inhibited. These results suggest that the deficiency of phosphate acquisition could be due to decreased phosphate transport. The influence of calcineurin on phosphate transport has already been observed in the renal type II Na/Pi co-transporter (Moz et al., 2004). Mice with genetic deletion of the calcineurin A $\beta$  gene had a marked decrease in type II Na/Pi mRNA levels and remarkably did not show the expected increase in type II Na/Pi mRNA levels after the challenge of a low-Pi diet. Recently, Sopko et al. (2006) identified the yeast calcineurin-responsive transcription factor Crz1p as a substrate for Pho85p, providing additional evidence for a link between the *PHO* pathway and calcineurin regulation.

Calcineurin is also essential for *C. albicans* survival in serum (Blankenship et al., 2003 and Sanglard et al., 2003). However, unlike *A. fumigatus*, it has been proposed that the absence of growth is due to (i) the fact that in the serum lacks one or more components necessary for the proliferation of the

mutant strain, and (ii) the presence of an active killing component in the serum. In *C. albicans*, addition of yeast extract or supraphysiologic levels of the vitamins thiamine and niacin rescued the growth of calcineurin mutants (Blankenship et al., 2003). Apparently, the mechanisms that underlie the absence of growth in serum in both biological systems could be different. However, we cannot completely discard the possibility that FBS contains another “inhibitory” factor that has a larger effect on growth of the already impaired mutant.

In summary, we have found that calcineurin is important for *A. fumigatus* morphogenesis, fitness, and growth in serum. The loss of fitness in the animal model and the inability to grow in FBS could be related to a combination of factors, such as decreased conidia and hyphae adhesion to the host tissue, and the nutritional deficiency to inorganic phosphate. Our work on calcineurin opens new venues for the research on sensing and nutrient acquisition in *A. fumigatus*.

#### Acknowledgments

This research was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil, and the Deutsche Forschungsgemeinschaft (SPP1160), Germany. We also thank Walter Richter and Renate Kaiser for helpful assistance in scanning electron microscopy, and the three anonymous reviewers for their comments.

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