

1 *Aspergillus fumigatus* *sitA* phosphatase homologue is important

2 for adhesion, cell wall integrity, biofilm formation and virulence

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29 **ABSTRACT**

30

31 *Aspergillus fumigatus* is an opportunistic pathogenic fungus able to infect
32 immunocompromised patients, eventually causing disseminated infections that
33 are difficult to control and lead to high mortality rates. It is important to
34 understand how the signalling pathways that regulate these factors involved in
35 virulence are orchestrated. Protein phosphatases are central to numerous
36 signal transduction pathways. Here we characterize *A. fumigatus* protein
37 phosphatase 2A SitA, the *S. cerevisiae* Sit4p homologue. The *sitA* gene is not
38 an essential gene and we were able to construct an *A. fumigatus* null mutant.
39 The $\Delta sitA$ strain had decreased MpkA phosphorylation levels, was more
40 sensitive to cell wall damaging agents, had increased β -1,3-glucan and chitin,
41 was impaired in biofilm formation, and had decreased protein kinase C activity.
42 The $\Delta sitA$ strain is more sensitive to several metals and ions such as $MnCl_2$,
43 $CaCl_2$, and $LiCl$, however, it is more resistant to $ZnSO_4$. The $\Delta sitA$ strain was
44 avirulent in a murine model of invasive pulmonary aspergillosis and induces an
45 augmented TNF- α response in mouse macrophages. These results stress the
46 importance of *A. fumigatus* SitA as a possible modulator of PkcA/MpkA activity
47 and its involvement in the cell wall integrity pathway.

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61 INTRODUCTION

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63 Human opportunistic pathogenic fungi are able to infect immunocompromised
64 patients causing disseminated infections that are difficult to control. Among
65 fungal human infections, invasive aspergillosis (IA) has one of the highest
66 mortality rates. Immunocompromised patients can acquire IA through the
67 inhalation of conidia that invade the pulmonary alveoli, translocating into the
68 blood and subsequently disseminating to other organs and niches throughout
69 the human body. *Aspergillus fumigatus* is the main causal agent of aspergillosis
70 (1,2,), causing around 65% of all *Aspergillus* invasive infections and is the most
71 encountered species in pulmonary infections (3,4,5). Aspergillosis is a
72 multifactorial disease with several phenotypes influencing the final outcome of
73 the disease. Recent advances have revealed several key pathogenicity
74 determinants involved in the establishment of the infection, such as the
75 composition of the cell wall, iron assimilation, hypoxia tolerance, gliotoxin
76 production and thermophily (1). However, it is important to understand how
77 these traits are orchestrated and the signaling pathways that regulate them
78 during virulence. *A. fumigatus* is able to alter its metabolism, secretome and cell
79 surface in order to adapt to the distinct microenvironments encountered within
80 the host, while also being able to bypass host defenses. Consequently, it is very
81 important to understand the signaling pathways and molecular mechanisms that
82 are involved in virulence, as this will provide new insights into the understanding
83 of this pathosystem and the development of new approaches to combat the
84 diverse range of diseases caused by this deadly pathogen.

85 Protein phosphorylation state regulates protein activity, localization and function
86 (6), with protein phospho-relays performing a crucial role in signal transduction.
87 Thus, protein kinases and phosphatases perform opposing functions in
88 numerous signal transduction cascades. Mitogen-activated protein (MAP)
89 kinase phosphorylation cascades are important for relaying, integrating and
90 amplifying intracellular signals and are crucial signaling components involved in
91 many cellular processes (7). In *Saccharomyces cerevisiae*, MAP kinases
92 control mating, the cellular response to high environmental osmolarity,
93 pseudohyphal development, sporulation of diploid cells and the maintenance of
94 cell wall integrity in response to stresses, such as heat stress and low

95 osmolarity (8). For instance, the *S. cerevisiae* cell wall integrity (CWI) pathway
96 is activated when Pkc1p phosphorylates Bck1p, a MAPKKK (a mitogen
97 activated protein kinase kinase kinase) which functions via the sequential
98 phosphorylation of two other protein kinases (MAPKK, MAPK) resulting in the
99 activation of a multifunctional MAP kinase (8). *S. cerevisiae* MAPKKs, Mkk1p
100 and Mkk2p, phosphorylate the MAPK Slf2p. Mkk1p/2p and Slf2p regulate the
101 expression of many downstream protein targets such as cell wall proteins and
102 enzymes involved in cell wall biogenesis (9).

103 In filamentous fungi the conserved MAPK pheromone response, filamentous
104 growth, osmotic stress response and cell wall integrity pathways have been
105 shown to influence numerous virulence traits including invasive growth, biofilm
106 formation, mycotoxin production and antifungal tolerance (8,10–14). *A.*
107 *fumigatus* has four MAPKs, MpkA (regulation of cell wall integrity signaling and
108 pyomelanin formation), MpkB (mating, putative pheromone signaling), MpkC
109 (regulation of conidial germination) and SakA (the Hog1 ortholog which is
110 involved in osmotic stress, carbon and nitrogen starvation, and regulation of
111 conidial germination) (15–20). The *A. fumigatus* CWI pathway is composed of
112 three MAP kinases designated Bck1, Mkk2 and MpkA. Deletion of these three
113 kinase encoding genes resulted in mutants with increased sensitivity to cell
114 wall-damaging agents and morphological alterations (18,19,21,22). The *A.*
115 *fumigatus* CWI pathway was further characterized by the identification of
116 putative CWI stress sensors Wsc1, Wsc3, and MidA, the Rho GTPase Rho1,
117 and Rom2, a guanine nucleotide exchange factor (23). Rom2 has been
118 positioned between Wsc1, Wsc3, MidA, and Rho1 and their downstream
119 effector MAP kinase module Bck1-Mkk2-MpkA (24).

120 In *S. cerevisiae* phosphatases, such as Msg5p and Sdp1p, dephosphorylate
121 Mpk1, contributing to the regulation of this signal transduction pathway (25).
122 Angeles de la Torre-Ruiz *et al.* (26) have reported that the correct down-
123 regulation of both basal and induced activity of the PKC1-MAPK pathway
124 requires the function of the Sit4p phosphatase. Sit4p is a type 2A-related
125 serine-threonine phosphatase that functions in the G1/S transition of the mitotic
126 cycle and is a nuclear protein that modulates functions mediated by Pkc1p
127 including cell wall and actin cytoskeleton organization (26–29). The protein

128 kinase TOR (Target of Rapamycin) promotes the phosphorylation of Sit4p by
129 negatively regulating Sit4p through its association with Tap42p. If TOR is
130 inactivated by rapamycin treatment or nitrogen starvation, downstream effectors
131 of TOR are dephosphorylated in a Sit4p-dependent manner (30). Recently,
132 aiming to comprehend the functions of protein phosphatases in *A. fumigatus*,
133 we identified 32 phosphatase catalytic subunit encoding genes (31). Within this
134 phosphatase null mutant collection was the *sitA* deletion strain, representing the
135 *S. cerevisiae* Sit4p homologue. The $\Delta sitA$ strain had decreased MpkA
136 phosphorylation, was more sensitive to cell wall damaging agents, had
137 increased β -1,3-glucan and chitin, reduced protein kinase C activity, and was
138 impaired in biofilm formation, while also being avirulent in a murine model of
139 invasive pulmonary aspergillosis. These results stress the importance of the *A.*
140 *fumigatus* SitA phosphatase as a possible modulator of PkcA/MpkA activity and
141 virulence.

142

143 MATERIALS AND METHODS

144

145 **Ethics statement.** The principles that guide our studies are based on the
146 Declaration of Animal Rights ratified by the UNESCO in January 27, 1978 in its
147 8th and 14th articles. All protocols used in this study were approved by the local
148 ethics committee for animal experiments from the Campus of Ribeirão Preto,
149 Universidade de São Paulo (Permit Number: 08.1.1277.53.6; Studies on the
150 interaction of *Aspergillus fumigatus* with animals). All animals were housed in
151 groups of five within individually ventilated cages and were cared for in strict
152 accordance with the principles outlined by the Brazilian College of Animal
153 Experimentation (Princípios Éticos na Experimentação Animal - Colégio
154 Brasileiro de Experimentação Animal, COBEA) and Guiding Principles for
155 Research Involving Animals and Human Beings, American Physiological
156 Society. All efforts were made to minimize suffering. Animals were clinically
157 monitored at least twice daily and humanely sacrificed if moribund (defined by
158 lethargy, dyspnoea, hypothermia and weight loss). All stressed animals were
159 sacrificed by cervical dislocation.

160

161 **Strains and media.** The *A. fumigatus* parental strains used in this study were
162 CEA17 (*pyrG*⁻) and CEA17-80. The *A. fumigatus* mutants constructed in this
163 study are Δ *sitA* and Δ *sitA::sitA*⁺. Media were of two basic types. A complete
164 medium with three variants: YAG (2% glucose, 0.5% yeast extract, 2% agar,
165 trace elements), YUU (YAG supplemented with 1.2 g/l each of uracil and
166 uridine) and liquid YG or YUU medium of the same composition (but without
167 agar). A modified minimal medium (MM: 1% glucose, original high nitrate salts,
168 trace elements, 2% agar, pH 6.5) was also used. Trace elements, vitamins, and
169 nitrate salts are described by (32).

170

171 **DNA manipulations and construction of the *A. fumigatus* mutants.** The
172 cassettes for gene replacement were constructed by *in vivo* recombination in *S.*
173 *cerevisiae* as previously described by (33). Approximately 1.5 kb from the 5'-
174 untranslated region (UTR) and 3'-UTR flanking region of the targeted genes
175 were selected for primer design. The primers 5F and 3R contained a short
176 sequence homologous to the multiple cloning site (MCS) of the pRS426
177 plasmid. Both the 5- and 3-UTR fragments were PCR-amplified from *A.*
178 *fumigatus* genomic DNA (gDNA). The *pyrG* inserted into the gene replacement
179 cassettes was amplified from pCDA21 plasmid and was used to generate a
180 marker for prototrophy in the mutant strains. Each fragment along with the
181 *Bam*HI/*Eco*RI cut pRS426 plasmid was transformed into the *S. cerevisiae* strain
182 SC9721 using the lithium acetate method (34). The transformant DNA was
183 extracted according to (31). The cassette was PCR-amplified from the plasmids
184 utilizing TaKaRa Ex Taq DNA Polymerase (Clontech Takara Bio) and used for
185 *A. fumigatus* transformation. Southern blotting was performed as described by
186 (35) aiming to demonstrate that the transformation cassettes had integrated
187 homologously at the targeted *A. fumigatus* loci. DNA fragments were labelled
188 with ³²P- α -dCTP using the RTS Rad Prime DNA labelling System kit
189 (Invitrogen).

190 A spontaneous *pyrG* mutant was selected from the deletion mutant strain by
191 using fluoorotic acid and this mutant was complemented by co-transforming
192 pCDA21 vector containing *A. fumigatus pyrG* gene plus the wild type *sitA* gene

193 and selecting for uracil and uridine prototrophy. The primers used above are
194 described in Table S1. All the Southern blots, PCRs, and the corresponding
195 strategies to evaluate if the phosphatase genes were deleted are shown in
196 Figure S1.

197

198 **Phenotypic assays.** The phenotypes of the deletion mutants were evaluated
199 either by radial growth or assessing the initial growth of a droplet of conidia from
200 a serial dilution, at different temperatures, in the presence or absence of
201 oxidative and osmotic stressing agents plus reagents that cause cell wall or
202 DNA damage. Drop out experiments were performed using five μ l of a tenfold
203 dilution series starting at a concentration of 2×10^7 for the wild-type and mutant
204 strains spotted on different growth media and grown for 48 h at 37°C.

205

206 **Fungal adhesion and biofilm formation assays.** The *A. fumigatus* crystal
207 violet assay and biofilm growth assays were performed according to (36,37),
208 respectively. In order to determine the ability to form biofilms 1×10^4 conidia were
209 inoculated into 200 μ l MM or YG liquid media within a 96 well polystyrene
210 microtiter plate, which was incubated, without agitation, at 37°C for 24h.
211 Subsequently, the plate was washed exhaustively with PBS prior to incubation
212 with 200 μ l 0.5% crystal violet solution for 5 mins at room temperature. The
213 stained mycelia were then exhaustively washed with sterile water and air dried.
214 Finally, crystal violet was eluted from the wells using 100 % ethanol and the
215 absorbance measured at 590 nm.

216 For biofilm formation, asexual spore suspensions (10^6 spores in total) were
217 added to 20 ml HEPES-buffered RPMI 1640 with L-glutamine (Life
218 Technologies) and dispensed on flat, presterilized, polystyrene Petri dishes.
219 Following an initial adherence phase of 4 h during static incubation in RPMI at
220 37°C, unbound conidia were washed three times with sterile Phosphate-
221 Buffered Saline (PBS) -0.1% Tween 80 solution (final concentration of PBS:
222 NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, and KH_2PO_4 1.8 mM). Fresh
223 RPMI medium with the indicated additives was added to the adhered conidia

224 and static submerged cultures were grown up to 96 h at 37°C. Mycelia were
225 harvested by scraping from the surface of the Petri dishes and filtrated through
226 Miracloth (Merck). The dry weight of biofilm produced was determined.

227

228 **Scanning electron microscopy.** Standardized conidia of *ΔsitA* and its parental
229 strain were inoculated in RPMI medium onto Thermanox coverslips (13 mm)
230 within a 24-well tissue culture plate. After 24h incubation at 37°C, biofilms were
231 processed and imaged as previously described (38). Briefly, the biofilms were
232 washed in PBS and fixed in 2% paraformaldehyde, 2% glutaraldehyde, and
233 0.15% [wt/vol] alcian blue in 0.15 M sodium cacodylate (pH 7.4). The biofilms
234 were sputter coated with gold and viewed under a JEOL JSM-6400 scanning
235 electron microscope in high vacuum mode at 10 kV.

236

237 **Transmission Electron microscopy (TEM).** Preparation of samples was as
238 previously described (39) with the following modifications. Briefly, cells were
239 collected and the pellets were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M
240 sodium phosphate buffer (pH 7.3) for 24 hr at 4°C. Samples were encapsulated
241 in 3% (w/v) low melting point agarose prior to processing to Spurr resin
242 following a 24 h schedule on a Lynx tissue processor (secondary 1% OsO₄
243 fixation, 1% Uranyl acetate contrasting, ethanol dehydration and infiltration with
244 acetone/Spurr resin). Additional infiltration was provided under vacuum at 60°C
245 before embedding in TAAB capsules and polymerising at 60°C for 48 h. Semi-
246 thin (0.5 μm) survey sections were stained with toluidine blue to identify areas of
247 best cell density. Ultrathin sections (60 nm) were prepared using a Diatome
248 diamond knife on a Leica UC6 ultramicrotome, and stained with uranyl acetate
249 and lead citrate for examination with a JEM-1400Plus transmission electron
250 microscope (JEOL (U.K.) LTD, Hertfordshire, UK) and imaging with an AMT
251 UltraVUE camera and AMT Image Capture Engine V602 software (Deben UK
252 Limited, Suffolk, UK).

253

254 **Staining for dectin-1 and chitin.** This procedure was performed as described
255 by (31,40). Briefly, *A. fumigatus* conidia were grown for 6 h at 37°C, UV-
256 irradiated, blocked using blocking solution (goat serum 2%, BSA 1%, 0.1%

257 Triton X-100, 0.05% Tween 20, 0.05% sodium azide and 0.01M PBS) for 1 h at
258 room temperature, and stained with conditioned medium containing 1 µg/ml of
259 s-dectin-hFc followed by DyLight 594-conjugated, goat anti-human IgG1 (41).
260 For chitin staining, UV-irradiated germlings were treated with CFW 2 µg/ml for 5
261 minutes. After washing, stained cells were visualized under identical imaging
262 conditions for parallel comparison using a Zeiss Observer Z1 fluorescence
263 microscope. Staining was quantified as the averaged amount of staining to the
264 total fungal area using ImageJ software.

265

266 **Analysis of conidia surface proteins.** Total proteins from the fungal conidial
267 surface were extracted as described by (42). Briefly, 2×10^9 conidia from three
268 independent samples of wild-type and $\Delta sitA$ were incubated with 200 µl of 0.5M
269 NaCl solution for 2 h at room temperature. The supernatant was collected by
270 centrifugation at 12 000 r.p.m. for 5 min, lyophilized, and resuspended in 50 µL
271 of buffer Tris-HCl 0.1M pH 8.8 with urea 8M. The proteins were quantified
272 using the Bradford method with Protein Assay Dye Reagent Concentrate (Bio-
273 Rad, cód. 500-0006, Lote L9700067 Rev J). The sample preparation for the
274 mass spectrometry consisted of three main steps: (i) protein reduction and
275 alkylation by adding 2.5 µL of dithiotreitol (DTT) in the ratio 1mg DTT/mg protein
276 and incubation for 2 h at room temperature followed by alkylation by adding 5
277 µL of iodoacetamide (IA) in the ratio 3 mg IA/mg protein and incubation for 1
278 hour at room temperature); (ii) enzymatic digestion of the proteins with trypsin.
279 The sample was diluted five-fold in 0.1 ammonium bicarbonate pH $\geq$ 8.0
280 yielding a final volume of 300 µL. The samples were then incubated with 1 µg
281 of trypsin (Promega, V511A, Lot 30551310) at 37°C for 16 h; and (iii) “clean
282 up/desalting” of the samples using the column OASIS HLB Cartridge 1cc (cat.
283 number: 186000383, Waters). The column was equilibrated with a solution
284 containing 5% acetonitrile, 0.1% formic acid, and the elution of the material was
285 performed with 80% acetonitrile. The samples were dried and applied into the
286 mass spectrometer LTQ Orbitrap ELITE (Thermo-Finnigan) coupled to a
287 nanoflow chromatography system (LC-MS/MS). The acquired data were
288 automatically processed by “Computational Proteomics Analysis System –
289 CPAS” (43). The identified peptides were grouped into proteins using the

290 algorithm “Protein Prophet” and a list of identified proteins was established
291 using an error lower than 2 %. The data were compared with *A. fumigatus*
292 proteomes (Af293 or A1163) at www.aspgd.org.

293

294 **Murine model of pulmonary aspergillosis.** The murine model of pulmonary
295 aspergillosis was performed according to (44). Outbred female mice (BALB/c
296 strain; body weight, 20 to 22 g) were housed in vented cages containing 5
297 animals. Mice were immunosuppressed with cyclophosphamide at a
298 concentration of 150 mg per kg of body weight, which was administered
299 intraperitoneally on days -4, -1, and 2 prior to and post-infection (day 0).
300 Hydrocortisone acetate (200 mg/kg) was injected subcutaneously on day -3.
301 The *A. fumigatus* conidia used for inoculation were grown on *Aspergillus*
302 complete YGA for 2 days prior to infection. Fresh conidia were harvested in
303 PBS and filtered through a Miracloth (Calbiochem). Conidial suspensions were
304 spun for 5 min at 3,000 × g, washed three times with PBS, counted using a
305 hemocytometer, then resuspended at a concentration of 5.0×10^6 conidia/ml.
306 Viability counts for the administered inoculum were determined, following serial
307 dilution, and plating on *Aspergillus* YGA, and the conidia were grown at 37°C.
308 Mice were anesthetized by halothane inhalation and infected by intranasal
309 instillation of 1.0×10^5 conidia in 20 µl of PBS. As a negative control, a group of
310 5 mice received PBS only. Mice were weighed every 24 h from the day of
311 infection and visually inspected twice daily. In the majority of cases, the
312 endpoint for survival experimentation was identified when a 20% reduction in
313 body weight was recorded, at which time the mice were sacrificed. The
314 statistical significance of comparative survival values was calculated using log
315 rank analysis using the Prism statistical analysis package. Additionally, at 3
316 days post-infection, 2 mice per strain were sacrificed and the lungs were
317 removed, fixed, and processed for histological analysis.

318

319 **Lung histopathology and fungal burden.** After sacrifice, the mice lungs were
320 removed and fixed for 24 h in 3.7% formaldehyde–PBS. Samples were washed
321 several times in 70% alcohol before dehydration in a series of alcohol solutions
322 of increasing concentrations. Finally, the samples were diafanized in xylol and
323 embedded in paraffin. For each sample, sequential 5- μ m-thick sections were
324 collected on glass slides and stained with Gomori methenamine silver (GMS) or
325 hematoxylin and eosin (HE) stain following standard protocols (2). Briefly,
326 sections were deparaffinized, oxidized with 4% chromic acid, stained with
327 methenamine silver solution, and counterstained with hematoxylin. Tissue
328 sections were also stained with hematoxylin and eosin for histological
329 examination to determine lung damage. All stained slides were immediately
330 washed, preserved in mounting medium, and sealed with a coverslip.
331 Microscopic analyses were performed using an Axioplan 2 imaging microscope
332 (Carl Zeiss) at the stated magnifications under bright-field conditions.

333 To investigate fungal burden in the lungs, mice were infected as described
334 previously, but with a higher inoculum of 1×10^6 conidia/20 μ l. A higher
335 inoculum, in comparison to the survival experiments, was used to increase
336 fungal DNA detection. Animals were sacrificed 72 h post-infection, and both
337 lungs were harvested and immediately frozen in liquid nitrogen. Samples were
338 homogenized by vortexing with glass beads for 10 min, and DNA was extracted
339 via the phenol-chloroform method. DNA quantity and quality were assessed
340 using a NanoDrop 2000 spectrophotometer (Thermo Scientific). At least 500 μ g
341 of total DNA from each sample was used for quantitative real-time PCRs. A
342 primer and a Lux probe (Invitrogen) were used to amplify the 18S rRNA region
343 of *A. fumigatus* (primer, 5'-CTTAAATAGCCCGGTCCGCATT-3'; probe, 5'-
344 CATCACAGACCTGTTATTGCCG-3') and an intronic region of mouse GAPDH
345 (glyceraldehyde-3-phosphate dehydrogenase) (primer, 5'-
346 CGAGGGACTTGAGGACACAG-3'; probe, 5'-
347 GGGCAAGGCTAAAGGTCAGCG-3'). Six-point standard curves were
348 calculated using serial dilutions of gDNA from all the *A. fumigatus* strains used
349 and the uninfected mouse lung. Fungal and mouse DNA quantities were
350 obtained from the threshold cycle (CT) values from an appropriate standard

351 curve. Fungal burden was determined as the ratio between picograms of fungal
352 and micrograms of mouse DNA.

353

354 **Phagocytosis index.** Alveolar macrophages (AM) were obtained from lungs of
355 of BALB/c mice (8 to 10 weeks old) by bronchoalveolar lavage (BAL) with 1-ml
356 of RPMI 1640 medium (Sigma-Aldrich) using an intravenous catheter (Becton
357 Dickinson) (18g) and pumping the medium into the lungs through the trachea.
358 Six mice per experiment were sacrificed. Macrophages from all six animals
359 were mixed and centrifuged at 4,000 rpm for 5 min. The supernatant was
360 removed, and the pellet was washed with 5 ml of RPMI and resuspended in 1
361 ml of RPMI–10% fetal bovine serum (FBS) (Gibco). AMs were counted in a
362 hemocytometer. The phagocytic assay was performed according to (44,45) with
363 some changes. Briefly, in a 24-well plate containing one 15-mm-diameter
364 coverslip per well, about 2×10^4 macrophages were incubated with 1 ml of
365 RPMI-FBS at 37°C with 5% CO₂ for 1 h. Afterwards, the wells were washed
366 with 1 ml of assay medium to remove non-adherent cells. Alveolar
367 macrophages are adherent and following this step the number of macrophage
368 cells is typically greater than 90% macrophages. In each well, 1 ml of RPMI-
369 FBS containing 1×10^5 conidia (1:5 macrophage/conidia ratio) was added.
370 Duplicate wells were assayed for each strain. The samples were incubated at
371 37°C with 5% CO₂ for 80 min, after which the supernatant was removed and
372 500 µl of 3.7% formaldehyde–PBS was added. After 15 min, the samples were
373 washed with 1 ml of ultrapure water and incubated for 20 min with 500 µl of
374 Calcofluor White (0.1 mg/ml). The samples were washed and mounted on
375 slides with 50% glycerol. A Zeiss Observer.Z1 fluorescence microscope was
376 used to assess the percentage of phagocytosed spores. Macrophage cells were
377 not permeable; hence, only internalized conidia remained unstained by
378 Calcofluor White. At least 100 conidia were counted per sample, and a
379 phagocytosis index was calculated. The experiments were repeated in triplicate.

380

381 **Conidial killing by alveolar macrophages.** To assess conidial killing by
382 alveolar macrophages, the phagocytic cells were obtained as described above.

383 In a 96-well plate, 1×10^4 macrophages were added with 200 μ l of RPMI-FBS
384 per well and incubated at 37°C with 5% CO₂ for 1 h. Afterwards, 5×10^4 conidia
385 (1:5 macrophage/conidia ratio) were added and incubated at 37°C with 5% CO₂
386 for 4 h. As a positive control, wells containing medium and conidia without
387 macrophages were used. Duplicate wells were assayed for each strain with and
388 without macrophages. After incubation, 100 μ l of 3% Triton X-100 was added.
389 After 10 min at 25 °C, samples were removed from the plate and serially diluted
390 in sterile saline. The dilutions were plated onto *A. fumigatus* complete medium
391 (44) and incubated at 37°C for 2 days. Conidial killing was calculated by
392 comparing colony forming units (CFU) numbers from samples incubated with
393 macrophages to CFU numbers from those incubated without macrophages. The
394 experiments were repeated three times.

395

396 **Determination of TNF- α levels.** For cytokine determination, Bone Marrow
397 Derived Macrophages (BMDMs) from C57BL/6 mice were prepared as
398 previously described (46). Briefly, bone marrow cells from femurs of adult mice
399 were cultured for 6 days in RPMI 1640, containing 20% fetal bovine serum
400 (FBS) and 30% L-929 cell conditioned media (LCCM). Macrophages (5.0×10^5)
401 were plated in 48-well plates for 16 h at 37°C, 5% CO₂ in RPMI 140 media
402 containing 10% FBS and 5% of LCCM. For fungal infection, strains were
403 cultured for 18 h up to a hyphal stage at a density of 2×10^4 per well, UV-
404 irradiated and used to stimulate the macrophages. The cells were centrifuged to
405 synchronize the infection and allowed to infect for 18 h. The supernatant was
406 collected and the cytokine was measured by enzyme-linked immunosorbent
407 assay (ELISA) with a mouse TNF- α kit (R&D Quantikine ELISA) according to
408 the manufacturer's instructions.

409

410 **Inhibition of PkcA, protein extraction and immunoblot analysis.** The
411 pharmacological inhibition of *A. fumigatus* PkcA was achieved by using
412 chelerythrine, calphostin C and cercosporamide. Conidia (1×10^6) of the wild-
413 type, $\Delta sitA$ and complementing strains were inoculated in 1 ml of liquid YG
414 medium in 24 well polystyrene plates containing 10% Alamar Blue (Life

Technologies) as the viability indicator, according to (47). Cells were grown for 48 h at 37°C and growth assessed after 24 h intervals. To assess the phosphorylation status of MpkA, fresh harvested conidia (1×10^7) of the wild-type and $\Delta sitA$ were inoculated in 50 ml liquid YG medium at 37°C for 16 h (180 rpm). After incubation, 1 μ g/ml of calphostin C was added to the cultures and incubated for an additional 30, 60, and 120 minutes. Control was left untreated. Mycelia were ground in liquid nitrogen with pestle and mortar. For protein extraction, 0.5 ml lysis buffer described in (48) containing 10% (v/v) glycerol, 50 mM Tris-HCl pH 7.5, 1% (v/v) Triton X-100, 150 mM NaCl, 0.1% (w/v) SDS, 5 mM EDTA, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 50 mM β -glycerophosphate, 5 mM sodium orthovanadate, 1 mM PMSF, and 1X Complete Mini® protease inhibitor (Roche Applied Science) was added to the ground mycelium. Extracts were centrifuged at 20,000 g for 40 minutes at 4°C. The supernatants were collected and the protein concentrations were determined using the Bradford method (49) (BioRad). 50 μ g of protein from each sample were resolved in a 12% (w/v) SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Merck Millipore). The phosphorylated fraction of the MAP kinase, MpkA, was examined using anti-phospho p44/42 MAPK antibody (9101; Cell Signaling Technologies) following the manufacturer's instructions using a 1:1000 dilution in TBST buffer (137 mM NaCl, 20 mM Tris, 0.1% Tween-20). Primary antibody was detected using an HRP-conjugated secondary antibody raised in rabbit (Sigma). Mouse anti γ -tubulin monoclonal antibody (yN-20; Santa Cruz Biotechnology) was used as loading control in the experiment. It was used in a 1:2500 dilution in TBST containing 3% skimmed milk. Anti γ -tubulin antibody was detected using a monkey peroxidase (HRP)-conjugated second antibody (Santa Cruz Biotechnology). Chemoluminescent detection was achieved using an ECL Prime Western Blot detection kit (GE HealthCare). Images were generated by exposing the membranes to the ChemiDoc™ XRS gel imaging system (BioRad). The images generated were subjected to densitometric analysis using ImageJ software (<http://rsbweb.nih.gov/ij/index.html>). The MapkA phosphorylated signal was normalized by γ -tubulin and the values of decreased phosphorylation upon calphostin C exposure in comparison to the untreated

448 controls were given as percentage. Detection of MpkA phosphorylation in
449 response to Congo Red (CR) stress was performed by growing the wild-type
450 and the $\Delta sitA$ mutant strains for 18 h at 37°C. Then, CR (300 µg/ml) was added
451 or not (control) for 15, 30, and 120 minutes.

452 Detection of SakA phosphorylation by Western blotting was performed as
453 described by (50) with slight modifications. Briefly, *A. fumigatus* conidia were
454 inoculated into liquid YPD (1% yeast extract, 1% polypeptone, and 1% glucose)
455 and cultured for 16 h prior to addition of 1/2 vol. 3 M sorbitol (Final
456 concentration: 1 M). Mycelia were harvested, frozen in liquid nitrogen, and
457 smashed in protein extraction buffer containing protease inhibitors. The
458 suspension was centrifuged and the supernatant was boiled with an appropriate
459 sample buffer. Proteins were separated with NuPAGE system (Invitrogen) and
460 blotted using an iBlot gel transfer system (Invitrogen). To detect SakA and
461 phosphorylated SakA proteins, a rabbit polyclonal IgG antibody against Hog1 y-
462 215 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a rabbit polyclonal
463 IgG antibody against dually phosphorylated p38 MAPK (Cell Signaling
464 Technology, Beverly, MA, USA) were used, respectively. To detect these
465 signals on blotted membranes, the ECL Prime Western Blotting Detection
466 System (GE Healthcare, Little Chalfont, UK) and LAS1000 (FUJIFILM, Tokyo,
467 Japan) were used.

468

469 RESULTS

470

471 Identification of the protein phosphatase SitA homologue in *A. fumigatus*.

472 A BLASTp search of the *A. fumigatus* genome revealed a single putative
473 orthologue of the *S. cerevisiae* Sit4 (ScSit4), Afu6g11470 (here named *sitA*).
474 The *sitA* gene model is supported by RNA-seq data (available at
475 www.aspgd.org) and three introns are predicted to be located at 155 to 211,
476 603 to 656, and 989 to 1036 bp. The hypothetical protein encoded by *sitA* was
477 predicted to be 388 amino acids in length and possessed a mass of 43.5 kDa.
478 The identity between the full length *A. fumigatus* SitA and ScSit4 proteins was
479 high (1e-99; 72.9% identity; 84.7% similarity). A comparison of protein structure

480 and organization between ScSit4 and SitA was performed using the SMART
481 interface (<http://smart.embl-heidelberg.de/>). The organization of the protein
482 phosphatase 2A catalytic domains was conserved in both proteins (data not
483 shown). Phylogenetic analyses identified numerous SitA homologues within
484 prominent filamentous fungi (protein identity greater than 70%), including
485 several *Aspergilli*, plant pathogens and saprophytic fungi (Figure 1). However,
486 the phylogenetic analyses clearly demonstrated two different branches from
487 yeast and molds (Figure 1).

488

489 **The *A. fumigatus* Δ sitA has impaired cell wall integrity.** To gain a better
490 insight into the function of SitA in *A. fumigatus*, the *sitA* gene was deleted
491 (Figure S1) and the phenotypes of the deletion strain compared to the wild-type
492 strain. The individual gene deletion was also complemented with the
493 corresponding wild-type gene aiming to confirm the occurrence of possible
494 secondary mutations during the construction of the deletion strain. Phenotypes
495 related to *S. cerevisiae* Δ sit4 (26,28,29,51) and which could be affected by the
496 absence of SitA, such as, sensitivity to rapamycin, high osmotic stress, cell wall
497 damaging agents, metals, and differential growth on poor nutritional conditions,
498 were investigated.

499 The Δ sitA strain demonstrated comparable radial growth on both minimal and
500 complete media when compared to the parental and complemented strains,
501 except for growth at 24 h in complete medium (Figure S2), suggesting SitA was
502 not important for growth under poor nutrient conditions. The Δ sitA strain was
503 more sensitive to several metals and ions such as MnCl_2 , CaCl_2 , and LiCl
504 (Figures 2A, B, and D); however, interestingly it was more resistant to ZnSO_4
505 plus EDTA 1 mM (ethylenediaminetetraacetic acid; aiming to chelate all the pre-
506 existing metals in the medium; Figure 2C). The Δ sitA strain was as sensitive to
507 rapamycin as the wild-type and complementing strains (Figure S3), and was
508 more sensitive to cell wall damaging agents, such as Congo Red (CR),
509 Calcofluor White (CFW), and Sodium Dodecyl Sulfate (SDS) (Figure 3A–C).

510 To determine if SitA was involved in the Mpk1 pathway in *A. fumigatus*, the
511 amount and phosphorylation state of Mpk1p homologue, MpkA, was determined
512 in the presence and absence of CR stress. The phosphorylation level of the

513 MpkA protein was determined using the anti-p44/42 MAPK antibodies directed
514 against phosphorylated MpkA (Figure 3D). In the wild-type strain, MpkA
515 phosphorylation levels increase 5.68-, 20.8-, and 19.2-fold post transfer to CR
516 300 µg/ml for 15, 30, and 120 minutes, respectively (Figure 3D). The $\Delta sitA$ has
517 about 3-fold higher MpkA phosphorylation levels than the wild-type strain in the
518 absence of CR (Figure 3D). However, the $\Delta sitA$ mutant demonstrated levels of
519 MpkA induction 0.88-, 5.45-, and 4.13-fold post transfer to CR 300 µg/ml for 15,
520 30, and 120 minutes (Figure 3D). The MpkA phosphorylation levels in the $\Delta sitA$
521 mutant were about 4- to 5-fold lower than the wild-type strain (Figure 3D).

522 This increased sensitivity to cell wall damaging agents could be due to
523 alterations in cell wall organization. Thus, fungal cell wall chitin levels were
524 assessed via CFW staining. The intensity of CFW staining per fungal area was
525 20% higher in $\Delta sitA$ mutant than wild-type strain (Figures 4A and B). Soluble
526 dectin-1 staining was used to identify difference in the content, or exposure, of
527 β -1,3 glucans on the surface of the fungal cell wall, in both the wild-type and
528 $\Delta sitA$ strains. The $\Delta sitA$ strain was shown to have a higher level of β -glucans
529 relative to wild-type and complemented $\Delta sitA::sitA^+$ strains. The intensity of
530 dectin-1 staining per fungal area was 40% higher in the $\Delta sitA$ mutant compared
531 to wild-type strain (Figures 4A and C). Collectively, these results suggest that
532 the surface of the $\Delta sitA$ cell had more abundant chitin and β -1,3 glucans
533 compared to wild-type strain. Differences in susceptibility to caspofungin (more
534 resistant) were observed in the $\Delta sitA$ (Supplementary Figure S4). Additional
535 evidence for a role of SitA in the organization of the cell wall was provided by
536 TEM analysis (Figure 5A). Untreated $\Delta sitA$ germlings grown in MM had about 3-
537 fold thicker cell walls compared to the wild-type and the complementing strains
538 (Figure 5B and Table S2). Further exposure of the $\Delta sitA$ germlings to CFW and
539 Congo Red did not change cell wall thickness (Figure 5B and Table S2). In
540 contrast, when wild-type and the complementing strains were exposed to CFW
541 and CR, they had about 2.5- to 3-fold increased cell wall thickness (Figure 5B
542 and Table S2). Taken together these results strongly indicate that SitA is
543 important for the *A. fumigatus* cell wall integrity pathway.

544

545 **The *A. fumigatus* Δ sitA has reduced adhesion properties.** The altered
546 composition of the cell wall could also alter the adhesion of conidia and/
547 mycelia to surfaces or other fungal cells. The Δ sitA conidia showed a drastic
548 alteration in hydrophobicity and reduction in the hydrophobin RodA content
549 (Figures 6A and B). The ability to form biofilms on solid surfaces was assessed.
550 Biofilm formation evaluated by crystal violet (CV) and biomass accumulation
551 was decreased about 40 to 60% and 20%, respectively (Figures 6C and D).
552 Scanning electron microscopy (SEM) of the mycelia revealed that SitA
553 influenced the cell surface and the extracellular matrix during biofilm formation
554 (Figure 7). The surface of the Δ sitA appeared smooth, which was in stark
555 contrast to the wild-type strain (Figure 7). Collectively, this demonstrates that
556 SitA influences the conidia and hyphal surface, impacting upon hydrophobicity,
557 adhesion and biofilm formation.

558 We also investigated the total cell surface proteins in the Δ sitA compared to
559 wild-type by extraction of cell surface proteins using 0.5 M NaCl (41). The
560 proteins extracted from the conidial surface from both strains were analysed by
561 LC-MS/MS (Table S3). We were able to identify in both strains 213 conidia
562 surface proteins (Table S3), but only 52 of them are differentially expressed in
563 both strains (Table 1). Twenty-two of these differentially expressed proteins are
564 either absent in the Δ sitA strain (11 proteins) but present in the wild-type, or
565 present in the Δ sitA strain (also 11 proteins) but absent in the wild-type (Table
566 1). Fifty percent of these proteins are enzymes and most of them are involved in
567 the remodeling of cell wall, such as NagA (*N*-acetylhexosaminosidase) and
568 Gel1 (1,3- β glucanosyltransferase, *gelA*) (Table 1). Forty percent are
569 hypothetical proteins and 10% adhesin-like proteins (Table 1). Fourteen of
570 these 52 proteins were more highly expressed in the Δ sitA mutant strain (Table
571 1). These proteins comprise 7 enzymes, 2 belonging to the translation
572 machinery and two allergens (Aspf13 and Aspf34; Table 1). Sixteen proteins
573 were more highly expressed in the wild-type strain: they are 8 enzymes, 1
574 related to the translation machinery, 1 toxin, 5 hypothetical, and 1 allergen,
575 Aspf1 (Table 1). These results imply that SitA is important for the proper
576 conidial surface protein distribution that is revealed by NaCl extraction.

577

578 **The Δ sitA mutant is more sensitive to protein kinase C inhibitors.** *S.*
579 *cerevisiae* Sit4p phosphatase is required for the correct down-regulation of both
580 basal and induced activity of the PKC1 (26). Calphostin C, chelerythrine, and
581 cercosporamide have been used as protein kinase C inhibitors in mammals and
582 in fungi (52-57). We hypothesize if Δ sitA is modulating the protein kinase C
583 activity, the susceptibility status of Δ sitA to these inhibitors would be altered.
584 The Δ sitA growth was dramatically inhibited by calphostin C, cercosporamide,
585 and chelerythrine (Figure 8A-C). This was further confirmed by a reduced MpkA
586 phosphorylation in the Δ sitA mutant compared to the wild-type strain when both
587 strains were exposed to calphostin C (Figure 8D). These results strongly
588 suggest that Δ sitA has reduced protein kinase C activity and it is probably
589 modulating the expression of protein kinase C.

590

591 **SitA is important for *A. fumigatus* virulence in a low dose murine**
592 **infection.** The importance of SitA to *A. fumigatus* pathogenicity was evaluated
593 in a neutropenic murine model of invasive pulmonary aspergillosis (Figure 9A).
594 Wild-type infection resulted in a 100% mortality after 12 days post-infection,
595 while Δ sitA infection resulted in a significantly reduced mortality rate, at
596 approximately 15% after 15 days post-infection (Figure 9A, $p < 0.0003$ and
597 $p < 0.0007$ for the comparison between the wild-type and the deletion mutant,
598 Log-rank, Mantel-Cox and Gehan-Breslow-Wilcoxon tests, respectively).
599 Virulence was restored in an independent strain that resulted from a single
600 ectopic reintegration of the wild-type *sitA* locus and there was no statistical
601 difference between the wild-type and the complemented Δ sitA::*sitA*⁺ strain
602 (Figure 9A, $p > 0.3$ and $p > 0.4$ for the comparison between the wild-type and the
603 complemented strain, Log-rank, Mantel-Cox and Gehan-Breslow-Wilcoxon
604 tests, respectively), directly linking the attenuation of Δ sitA virulence to SitA
605 function.

606 Histopathological examination revealed that at 72 h post-infection the lungs of
607 mice infected with the wild-type strain contained multiple foci of invasive hyphal
608 growth, which penetrated the pulmonary epithelium in major airways, while
609 pockets of branched invading hypha originated from the alveoli (Figure 9B). In
610 contrast, Δ sitA infections revealed inflammatory infiltrates in bronchioles, with

611 some containing poorly germinated or ungerminated conidia (Figure 9B).
612 Fungal burden was measured by qPCR, showing that the $\Delta sitA$ strain did not
613 grow within the lungs as well as the wild-type and the complemented
614 $\Delta sitA::sitA^+$ strains (Figure 9C). Taken together, this strongly indicates that SitA
615 plays an important role in *A. fumigatus* virulence.

616 The impaired $\Delta sitA$ CWI together with the dramatic attenuation in virulence
617 could contribute to an altered immune response. Alveolar Macrophages (AM)
618 play an essential role in clearing *A. fumigatus* conidia from the lung (58).
619 Approximately 15% *A. fumigatus* wild-type and $\Delta sitA::sitA^+$ conidia were
620 internalized after 80 min of incubation with AM (Figure 10A). In contrast, about
621 40 % of $\Delta sitA$ conidia were internalized after the same period (Figure 10A).
622 After a 4 h incubation, no differences in the *in vitro* killing of resting conidia for
623 *A. fumigatus* wild-type, $\Delta sitA$, $\Delta sitA::sitA^+$ strains was observed (data not
624 shown). This data suggests that $\Delta sitA$ strain is more sensitive to phagocytosis,
625 while there are no differences in the AM killing for all three strains.
626 Subsequently, the cytokine Tumour Necrosis Factor alpha (TNF- α) levels
627 released from Bone Marrow Derived Macrophages (BMDMs) after co-incubation
628 with *A. fumigatus* hyphae were investigated. TNF- α is an important
629 inflammatory mediator secreted by macrophages when exposed to *A. fumigatus*
630 (59,60). BMDMs co-cultured with $\Delta sitA$ show higher TNF- α production than the
631 wild-type or the complemented strain (about 2.4-fold, Figure 10B). These
632 results suggest that the effect caused by SitA on the CWI is important for
633 macrophage recognition and inducing inflammatory responses.

634

635 DISCUSSION

636

637 *A. fumigatus* is a cosmopolitan fungus and is able to live and survive in different
638 environments. This ability demands it to possess great metabolic versatility and
639 the capability to adapt to these diverse ecological niches, through the precise
640 regulation of a complex network of signaling cascades and their downstream
641 effects. *A. fumigatus* efficiently utilizes the human body as a niche, causing
642 several clinical forms of disease, depending of the status of the human immune

643 system. How *A. fumigatus* is able to overcome host defenses and establish
644 infection is dependent on the coordination of these signal transduction
645 pathways. An enhanced understanding of these mechanisms of adaptation may
646 have impacts on the establishment of strategies to control the disease.
647 Recently, as an initial step to comprehend the signaling pathways regulating
648 this program of adaptation, we methodically investigated all the 32 *A. fumigatus*
649 phosphatase encoding genes and constructed a collection of 24 null mutants for
650 these genes (31 and unpublished results). Here we focus on a single
651 phosphatase, SitA, in greater detail, and assigned a role in cell signaling and
652 virulence.

653 The *A. fumigatus* gene *sitA* codes for a Ser/Thr protein phosphatase member of
654 the PPP phosphatase family that is closely related to the PP2A family. In *S.*
655 *cerevisiae*, Sit4p plays a critical role in cell growth and proliferation and is
656 involved in several biological processes, such as the TOR pathway-mediated
657 response to nutrients (61-64), the regulation of monovalent ion homeostasis
658 and intracellular pH (65), cell cycle regulation (28,66), is required for proper
659 telomere function (67), the initiation of translation (68), and the CWI pathway
660 (26). We have observed that the *A. fumigatus sitA* null mutant has neither
661 growth defects nor developmental problems. The *S. cerevisiae* Sit4p is
662 phosphorylated by TOR, negatively regulating Sit4p through the association of
663 Sit4p with Tap42p. The main signals for Sit4p activation are the treatment with
664 rapamycin or nitrogen starvation (30). When Sit4p is activated downstream
665 effectors of TOR are dephosphorylated in a Sit4p-dependent manner (30). The
666 *A. fumigatus* $\Delta sitA$ strain was not more sensitive to rapamycin than the
667 wild-type strain and we have also not observed any nutritional impairment in
668 this strain. Sit4 homologues have been characterized only in two other fungi,
669 *Candida albicans* and *F. graminearum* (62,69). In *C. albicans* SIT4 homologue
670 disruption resulted in reduced growth rate and virulence in a mouse infection
671 model (69). These authors observed that the *C. albicans* Sit4p was involved in
672 hyphal growth via regulating cell wall biogenesis, osmosensing and protein
673 translation. More recently, Sit4p was identified as a potential regulator of the
674 early hypoxic response in *C. albicans* (70). Previous to this work, there was only
675 one Sit4 homologue described in filamentous fungi, FgSIT4 in *F. graminearum*
676 (62). In the plant pathogen *F. graminearum*, FgSit4 plays important roles in

677 regulation of various cellular processes, including mycelia growth, virulence and
678 sexual development (62). FgSit4 affects the cell wall integrity pathway by
679 positively regulating the phosphorylation of FgMgv1, the key MAP kinase in the
680 CWI pathway.

681 The *A. fumigatus* cell wall is mainly composed by polysaccharides, such as
682 branched β -(1,3)-glucan to which chitin, β -(1,3)- β -(1,4)-glucan and
683 galactomannan are covalently bound, and α -(1,3)-glucan that has adhesive
684 property and stabilizes the cell wall (42). The conidial cell wall is covered by an
685 outer layer of rodlets and melanin, which possess hydrophobic properties as
686 well as being immunological inert (71–73). The outer layer has
687 galactosaminogalactan, while the mycelium grows embedded in an extracellular
688 matrix rich in polysaccharides, hydrophobins, and melanin (42). We have
689 observed that the *A. fumigatus* $\Delta sitA$ conidia have reduced hydrophobin
690 content, while this mutant is sensitive to cell wall damaging agents, and has
691 increased MpkA phosphorylation. Moreover, *A. fumigatus* $\Delta sitA$ germlings
692 altered cell wall organization, and the hyphae have reduced adhesion, biofilm
693 formation and extracellular matrix production. The conidia surface protein profile
694 of the $\Delta sitA$ mutant is also distinct from the wild-type. However, quantitative
695 analyses of cell wall components in $\Delta sitA$ stay for future investigation. All these
696 defects strongly suggest defects in the CWI pathway. The protein kinase C-
697 mediated mitogen-activated protein kinase (PKC1-MAPK) pathway is essential
698 for the activation of the CWI in fungi (12,74). The *S. cerevisiae* *SIT4 Δ* strain has
699 a G₁ to S delay in the cell cycle, which is mediated by the up-regulation of Pkc1
700 activity (26). In *S. cerevisiae*, Sit4 operates downstream of the plasma
701 membrane sensors Mid2, Wsc1, and Wsc2 and upstream of Pkc1, affecting
702 Pkc1 functions, such as Mpk1 activity and CWI, actin cytoskeleton organization,
703 and ribosomal gene transcription (26). It remains to be investigated in *A.*
704 *fumigatus* if SitA is modulating directly or indirectly PkcA, or other targets that
705 are involved in the CWI pathway. We have observed that $\Delta sitA$ mutant has
706 reduced MpkA phosphorylation when exposed to cell wall damaging agents and
707 is more sensitive to protein kinase C inhibitors, suggesting the PkcA activity in
708 this mutant is lower than in the wild-type strain. This is further emphasized by
709 the reduced MpkA phosphroylation in this mutant in the presence of these

710 inhibitors. It remains to be investigated in *A. fumigatus* if SitA is modulating
711 directly or indirectly PkcA, or other targets that are involved in the CWI pathway.

712 We have shown that $\Delta sitA$ has attenuated virulence and is increasingly
713 recognition by macrophages. In the immunosuppressed murine model of
714 invasive pulmonary aspergillosis, $\Delta sitA$ is avirulent when compared to the
715 wild-type and complemented strains. TNF- α , one of the key inflammatory
716 mediators secreted by macrophages in response to fungal hyphae, was
717 increased in $\Delta sitA$ compared to wild-type and complemented strain. This
718 pro-inflammatory cytokine plays an important role in the induction of the innate
719 immune response to *A. fumigatus* (60,67). The increased β -1,3 glucan, reduced
720 hydrophobin content, and other modifications in the $\Delta sitA$ cell wall could
721 contribute to an increased recognition of the fungus by dectin receptor, favoring
722 its increased phagocytosis by alveolar macrophages, and consequently the
723 increased TNF- α production, since β -glucan is a potent stimulator of the TNF- α
724 response in fungi (75–78).

725 In summary, we have identified that SitA is an important phosphatase for cell
726 wall construction and is essential for virulence and macrophage recognition. It
727 remains to be investigated how SitA is affecting the organization of the cell wall.
728 However, based on *S. cerevisiae* studies, it is likely that SitA is modulating the
729 PkcA activity. This improved understanding of the function of SitA in *A.*
730 *fumigatus* may in turn provide an opportunity to improve our knowledge of how
731 *A. fumigatus* regulates cell wall integrity and composition during virulence.

732

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741

742

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1036 **FIGURE LEGENDS**

1037

1038 Figure 1 – Phylogenetic tree of the fungal SitA homologues. The optimal tree for
1039 the *A. fumigatus* and *S. cerevisiae* phosphatases is represented. The tree was
1040 inferred using the Neighbour-Joining Method. The bootstrap values calculated
1041 on 500 replicates are indicated on the tree branches. Sequences were aligned
1042 with ClustalW and the tree was constructed by using MEGA6 software.

1043

1044 Figure 2 – *A. fumigatus* $\Delta sitA$ is more sensitive to ionic stress. *A. fumigatus*
1045 wild-type, $\Delta sitA$, and $\Delta sitA::sitA^+$ were grown for 48 h at 37 °C in MM+different
1046 concentrations of $MnCl_2$ (A), MM+different concentrations of $CaCl_2$ (B),
1047 MM+different concentrations of $ZnSO_4$ +1 mM of EDTA, or (D) MM+different
1048 concentrations of LiCl.

1049

1050 Figure 3 – *A. fumigatus* $\Delta sitA$ has impaired cell wall integrity. *A. fumigatus* $\Delta sitA$
1051 is more sensitive to cell wall damaging agents such as Congo Red (CR) and
1052 Calcofluor White (CFW). Conidial tenfold dilution (from 10^5 to 10^2) from wild-
1053 type, $\Delta sitA$, and $\Delta sitA::sitA^+$ mutant strains was plated on Minimal (A) and
1054 Complete (B) media. *A. fumigatus* $\Delta sitA$ is more sensitive to SDS (C).
1055 Immunoblot analysis for MpkA phosphorylation in response to CFW stress (D).
1056 The wild-type and the *sitA* null mutants were grown for 18 h at 37°C. Then, CR
1057 (300 µg/ml) was not added (control) or added for 15, 30, and 120 minutes. Anti-
1058 phospho-p44/42 MAPK antibody directed against phosphorylated MpkA was
1059 used to detect the phosphorylation of MpkA. Anti- γ -tubulin antibody was used
1060 as a control for loading. A Coomassie Brilliant Blue (CBB)-stained gel is shown
1061 as an additional loading control. Signal intensities were quantified using the
1062 Image J software by dividing the intensity of MpkA-P/ γ -tubulin.

1063

1064 Figure 4 – Detection of the β -1,3–glucan and chitin content on the cell surface.
1065 Conidia were cultured in liquid media to the hyphal stage, UV-killed, and stained

1066 with soluble dectin-1 or Calcofluor white (A) to detect the content of exposed
1067 chitin (B) or β -glucan (C) respectively. The intensity of staining was calculated
1068 by averaging the amount of staining to the total area of each fungal cell using
1069 ImageJ software. These experiments were performed in triplicate, and the
1070 results are displayed as mean values with standard errors (*= $p < 0.05$ by t-
1071 tests). Bars, 5 μ m.

1072

1073 Figure 5 – Transmission Electron Microscopy (TEM) for *A. fumigatus* wild-type,
1074 $\Delta sitA$, and $\Delta sitA::sitA^+$ mutant strains. Germlings were grown in the absence or
1075 presence of CFW (Calcofluor White) or Congo Red. (A) TEM analysis (bars,
1076 100 nm). (B) Cell wall thickness (μ m) of fifty sections of different germlings were
1077 measured (Table S2). The results are expressed as average $\pm$ standard
1078 deviation.

1079

1080 Figure 6 – *A. fumigatus* $\Delta sitA$ has reduced adhesion properties. (A) The fungal
1081 adhesion and biofilm formation is influenced by *SitA*. Wild-type and $\Delta sitA$
1082 conidia into 1:1 water/oil (tributyrin) interface. (B) Polyacrylamide gel showing
1083 the hydrophobin concentration of the wild-type, $\Delta sitA$ and $\Delta sitA::sitA^+$ conidia.
1084 RodAp corresponds to the native RodA and RodAp* to partially degraded or
1085 processed RodA. (C) Adhesion measured by Crystal Violet (CV) assay is
1086 reduced in the $\Delta sitA$ mutant in both 0.1% and 1% glucose (*, p -value < 0.01
1087 when compared to the wild-type strain). (D) Biofilm formation is reduced in the
1088 $\Delta sitA$ mutant.

1089

1090 Figure 7 – Scanning Electron Microscopy for the wild-type and $\Delta sitA$ mutant
1091 strains. The magnification is 1000 times and arrow head indicates extracellular
1092 matrix.

1093

1094 Figure 8 – The $\Delta sitA$ has reduced protein kinase activity. (A-C) Viability of the
1095 germlings of the wild-type, $\Delta sitA$, and $\Delta sitA::sitA^+$ grown in the absence or

1096 presence of calphostin C (nM), cercosporamide ($\mu\text{g/ml}$), and chelerythrine (μM)
1097 as shown by viability indicator Alamar Blue. The germlings are less viable when
1098 the indicator shows intense blue colonies indicating decreased mitochondrial
1099 activity. Pink and red colours show viability. (D) Western blot for MpkA
1100 phosphorylation. Wild-type and ΔsitA strains were grown for 16 h at 37 °C and
1101 then mycelia was transferred to fresh medium with calphostin C 1000 nM for 30,
1102 60, and 120 minutes. Anti-phospho-p44/42 MAPK antibody directed against
1103 phosphorylated MpkA was used to detect the phosphorylation of MpkA. Anti- γ -
1104 tubulin antibody was used as a control for loading. A Coomassie Brilliant Blue
1105 (CBB)-stained gel is shown as an additional loading control. Signal intensities
1106 were quantified using the Image J software by dividing the intensity of MpkA-
1107 P/ γ -tubulin.

1108

1109 Figure 9 – *A. fumigatus* ΔsitA contributes to virulence in neutropenic mice.(A)
1110 Comparative analysis of wild-type and mutant strains in a neutropenic murine
1111 model of pulmonary aspergillosis. Mice in groups of 10 per strain were infected
1112 intranasally with a 20 μl suspension of conidiospores at a dose of 10^5 . (B)
1113 Histological analysis of infection murine lung was performed 72 h after infection.

1114

1115 Figure 10 – Macrophage recognition and TNF- α secretion from bone marrow-
1116 derived macrophages (BMDMs). (A) There is an increase uptake of ΔsitA
1117 conidia by Alveolar Macrophages (AM) phagocytes. Percentage of conidia
1118 taken by AM. Number of conidia phagocytosed is increased for ΔsitA . Data show
1119 average $\pm$ standard deviation and $*p < 0.01$ compared to the wild-type and the
1120 complemented strain. (B) ΔsitA hyphae trigger significantly increase release of
1121 TNF- α from BMDMs compared to wild-type and the reconstituted strain.
1122 BMDMs from C57BL/6 mice were infected with *A. fumigatus* hyphae for 18h and
1123 the supernatant of cells was collected to measure the TNF- α levels by ELISA.
1124 Data show average $\pm$ standard deviation and $*p \leq 0.005$ compared to the
1125 wild-type and the complemented strain. NI = Non-infected.

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1147 Table 1 – *Conidia* surface proteins identified in the wild-type and $\Delta sitA$ mutant strains.

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<i>A.fumigatus</i> protein	Function	Mass (kDa)	Length	Ratio ^a $\Delta sitA$ /WT ^b	% coverage mutant	% coverage Wild-type
Conidia surface proteins absent in the $\Delta sitA$ mutant strain						
Afu1g17410	Beta-glucosidase	84	782	Only in WT	NI	2
Afu3g14030	Alkaline phosphatase, phoB-regulated	72	653	Only in WT	NI	17
Afu2g00690	Glucan 1,4-alpha-glucosidase activity, role in polysaccharide metabolic process and Golgi apparatus, endoplasmic reticulum, prospore membrane localization	67	631	Only in WT	NI	6
Afu2g17530	Laccase involved in conidial pigment biosynthesis	65	587	Only in WT	NI	15
Afu7g05140	Putative class III family 18 chitinase	46	448	Only in WT	NI	16
Afu3g00270	Cell wall glucanase; predicted glycoposphatidylinositol (GPI)-anchor; secreted protein	44	450	Only in WT	NI	5
Afu2g05240	Ortholog(s) have extracellular region localization	42	400	Only in WT	NI	11
Afu2g00680	ORF, uncharacterized; Has domain(s) with predicted catalytic activity	40	375	Only in WT	NI	8
Afu7g05650	ORF, uncharacterized; Ortholog of <i>A.fumigatus</i> A1163 : AFUB_091230	23	225	Only in WT	NI	8
Afu5g01420	ORF, uncharacterized; Ortholog(s) have extracellular region localization	22	205	Only in WT	NI	30
Afu2g13600	Pyruvate dehydrogenase (acetyl-transferring) kinase activity, role in carbon utilization, peptidyl-serine phosphorylation and mitochondrion localization	20	187	Only in WT	NI	8
Conidia surface proteins absent in the wild-type strain						
Afu8g05020	NagA, secreted N-acetylhexosaminidase; highly expressed in biofilm	67	600	Only in $\Delta sitA$	8	NI
Afu2g02100	dihydrolipoamide dehydrogenase; reacts with rabbit immunosera exposed to <i>A. fumigatus</i> conidia	54	513	Only in $\Delta sitA$	27	NI
Afu2g01170	Gel1, 1,3-beta-glucanosyltransferase with a role in elongation of 1,3-beta-glucan chains; glycosylphosphatidylinositol (GPI)-anchored protein; constitutively expressed during hyphal growth; hypoxia induced protein	48	452	Only in $\Delta sitA$	6	NI
Afu7g05740	NAD-dependent malate dehydrogenase; protein abundant in conidia; immunoreactive; reacts with rabbit immunosera exposed to <i>A. fumigatus</i> conidia; induced by L-tyrosine; transcript downregulated in response to hypoxia	35	340	Only in $\Delta sitA$	17	NI
Afu6g10930	ORF, uncharacterized; Protein of unknown function	30	290	Only in $\Delta sitA$	8	NI
Afu6g14370	AraC-like ligand binding domain protein	23	213	Only in $\Delta sitA$	9	NI
Afu3g00880	Putative adhesin protein; MedA regulated transcript; transcript repressed by exposure to human	21	219	Only in $\Delta sitA$	13	NI

	airway epithelial cells					
Afu3g09390	Laminin-binding protein with extracellular thaumatin domain; predicted adhesin-like protein; expression induced in biofilm; repressed by exposure to artemisinin	18	177	Only in $\Delta sitA$	14	NI
Afu1g01980	ORF, uncharacterized; Ortholog(s) have role in hyphal growth, response to cold, response to heat, response to oxidative stress, response to salt stress, sporocarp development involved in sexual reproduction	18	179	Only in $\Delta sitA$	6	NI
Afu5g01990	ORF, uncharacterized; BYS1 domain protein	16	156	Only in $\Delta sitA$	12	NI
Afu4g09320	DppIV, extracellular dipeptidyl-peptidase; predicted signal sequence for secretion; induced by growth on BSA as a sole nitrogen source; expression dependent on PrtI	85	765	30.5	18	NI
Conidia surface proteins more expressed in the $\Delta sitA$ mutant strain						
Afu5g10490	Amidase; secreted protein; fibrinogen-binding	65	611	18.8	5	3
Afu5g03540	Ortholog(s) have flavin-linked sulfhydryl oxidase activity, role in oxidation-reduction process and extracellular region localization	42	386	11.7	12	14
Afu4g13750	Mep20, penicillolysin/deuterolysin metalloprotease; predicted signal sequence for secretion	39	370	9.6	5	5
Afu1g06390	Tef1, translation elongation factor EF-1 alpha subunit; protein abundant in conidia; protein induced by heat shock	53	494	4.9	14	3
Afu3g08290	Aspartyl aminopeptidase; immunoreactive; secreted protein; induced by growth on BSA as a sole nitrogen source	54	504	3.9	6	5
Afu8g00710	ORF, uncharacterized; Has domain(s) with predicted role in defense response, negative regulation of growth	10	101	3.2	34	48
Afu2g09030	DppV, secreted dipeptidyl-peptidase; induced by growth on BSA as a sole nitrogen source; immunoreactive protein	79	721	2.9	24	12
Afu7g07010	Adh1, alcohol dehydrogenase; predicted gene pair with AFUA_5G06240 (alcC); induced by L-tyrosine; transcript up-regulated in conidia exposed to neutrophils	37	353	2.0	5	4
Afu5g09240	Sod1, Cu/Zn superoxide dismutase; recognized by sera from aspergillosis patients; highly expressed in conidia; transcript induced by copper starvation, menadione, gliotoxin and growth at high temperature; protein induced by hydrogen peroxide	15	154	2.0	28	30
Afu3g02270	Cat1, mycelial catalase; induced in hyphae exposed to neutrophils; protein induced in amphotericin B and H ₂ O ₂ ; stuA-dependent up-regulation in developmentally competent hyphae; hypoxia repressed; SrbA-regulated; repressed by gliotoxin exposure	79	728	2.0	22	21
Afu5g01120	ORF, uncharacterized; Ortholog of A. nidulans FGSC A4 : AN8927, A. oryzae RIB40 : AO090003001298, Aspergillus flavus NRRL 3357 : AFL2T_01773 and Neosartorya fischeri NRRL 181 : NFIA_041050	36	354	1.8	4	9
Afu2g12630	Asp f13, allergen Asp f13; putative alkaline serine protease; higher expression in biofilm vs planktonic cells; transcript induced by growth on hydrogen peroxide	15	152	1.8	43	44
Afu3g03060	Asp f34, allergen Asp f34; putative phiA family cell wall protein; induced calcium; repressed by exposure to artemisinin	19	185	1.8	9	9
Afu1g12070	ORF, uncharacterized; Ortholog(s) have glycine dehydrogenase (decarboxylating) activity, role	18	175	1.6	9	9

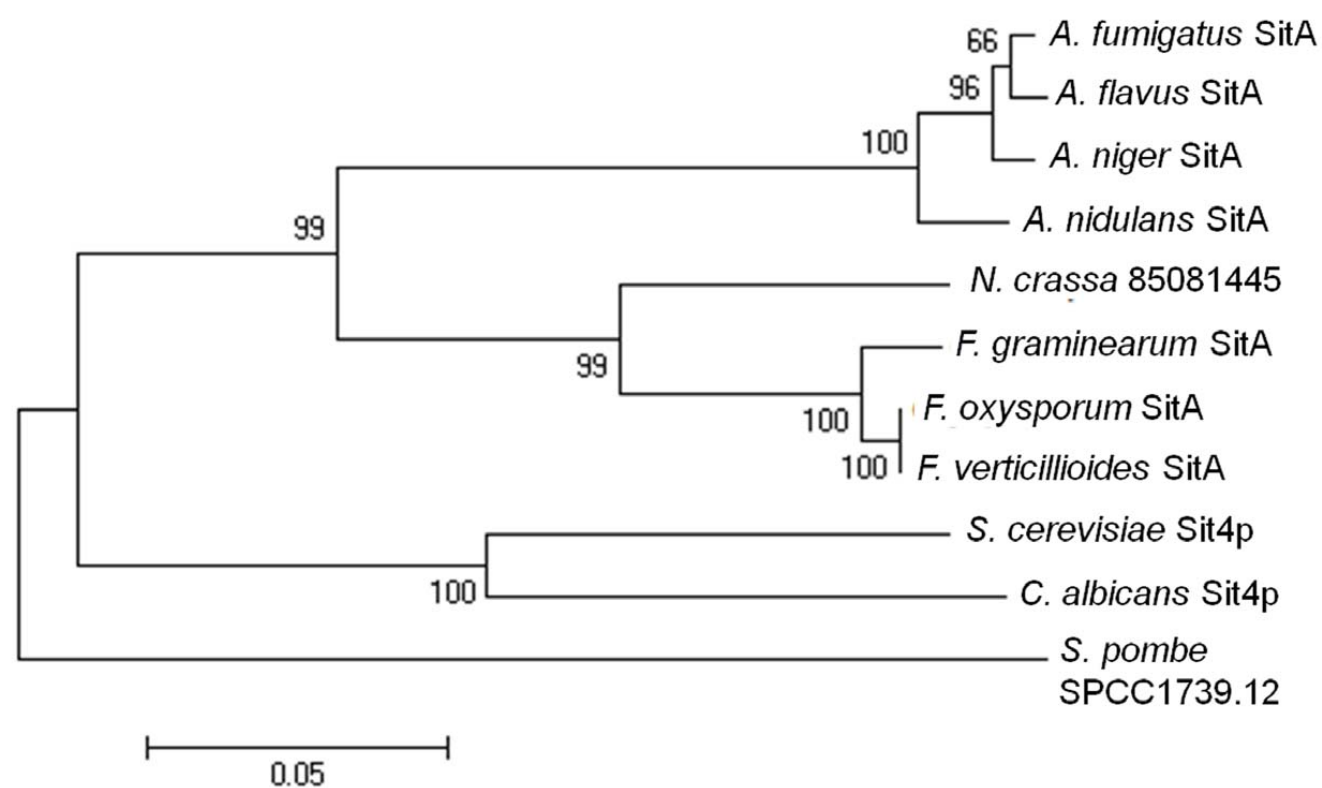
	in glycine catabolic process, one-carbon metabolic process, protein lipoylation and mitochondrion localization					
Afu3g15090	Adenosine deaminase family protein; secreted protein; predicted secretory signal sequence; ortholog of A. nidulans AN2494	66	587	1.3	16	15
Conidia surface proteins more expressed in the Wild-type strain						
Afu1g15730	40S ribosomal protein S22	14	130	0.9	11	11
Afu1g14450	ExgO, exo-beta-1,3-glucanase; secreted protein; predicted secretory signal sequence; ortholog of A. nidulans AN0779	10	947	0.8	10	13
Afu3g07030	Gla1, glutaminase A; secreted protein; predicted secretory signal sequence	76	691	0.8	16	20
Afu3g00590	AspHS, Asp-hemolysin; hemolytic toxin; highly secreted; enriched in conidia; expression increases in vivo; binds lysophosphatidylcholine	15	139	0.7	20	25
Afu1g05770	Exg12, secreted beta-glucosidase; predicted secretory signal sequence; fibrinogen-binding	94	873	0.6	10	20
Afu1g10970	Aalpha-1,2-mannosidase family protein	92	839	0.6	2	19
Afu6g14470	Ortholog of A. nidulans FGSC A4 : AN5353, A. niger CBS 513.88 : An02g05680, A. oryzae RIB40 : AO090103000108, Aspergillus wentii : Aspwe1_0131073 and Aspergillus sydowii : Aspsy1_0050288	26	234	0.5	10	18
Afu2g00967	Ortholog of A. nidulans FGSC A4 : AN7635/binA, AN11904, A. fumigatus Af293 : Afu3g02216, Afu7g00610 and A. niger CBS 513.88 : An10g00560	21	196	0.5	7	8
Afu6g03210	ConJ, protein of unknown function; conidia-enriched protein; ortholog of Neurospora crassa	87	83	0.4	35	26
Afu8g00630	uncharacterized; Ortholog(s) have extracellular region localization	37	347	0.3	27	41
Afu4g09280	ORF, uncharacterized; Ortholog of A. niger CBS 513.88 : An02g00330, Neosartorya fischeri NRRL 181 : NFIA_106720, Aspergillus niger ATCC 1015 : 36613-mRNA and Aspergillus carbonarius ITEM 5010 : Acar5010_203109	18	167	0.2	28	54
Afu4g11800	Alp1, secreted alkaline serine protease; cleaves human complement components C3, C4, and C5; predicted signal sequence for secretion; fibrinogen-binding	42	403	0.2	12	22
Afu6g12070	FmqD, Flavoprotein amide oxidase (FAD); has FAD activity; member of the fumiquinazoline biosynthetic cluster; may contain an N-terminal signal sequence and multiple N-glycosylation sites; transcript downregulated in response to voriconazole	54	497	0.2	5	14
Afu1g14560	MsdS, 1,2-alpha-mannosidase; secreted protein; fibrinogen-binding	53	493	0.2	2	7
Afu7g06140	Exg13, secreted 1,4-beta-D-glucan glucanhydrolase	78	739	0.1	2	7
Afu5g02330	Aspf1, Allergen Asp f 1; ribonuclease mitogillin family of cytotoxins; similar to restrictocin; inhibits protein synthesis in mammalian cells; 18kD IgE-binding protein; expression increases in vivo; biofilm-induced vs planktonic cells	19	176	0.05	7	17

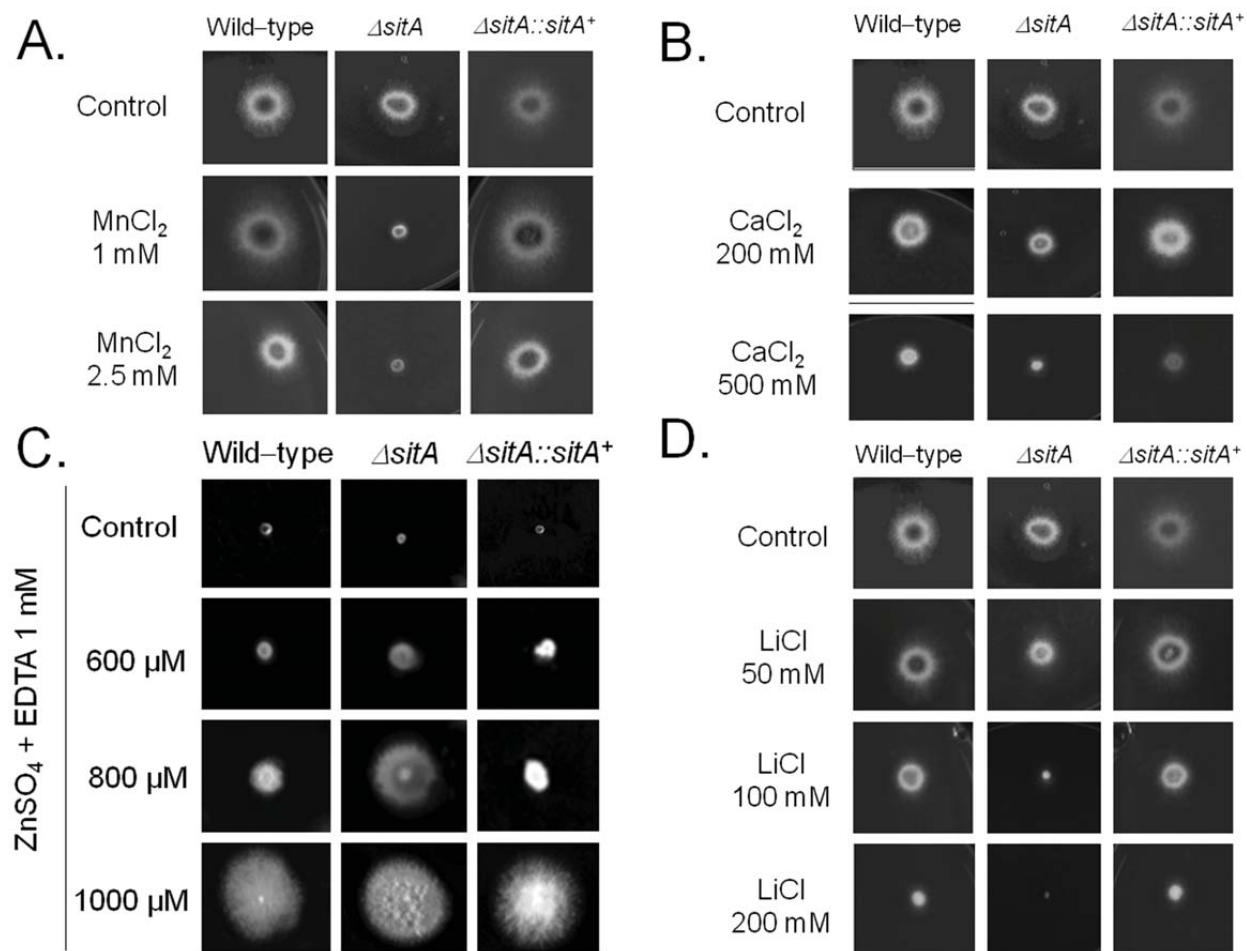
1149 ^aThe values are related to spectral counts of three repetitions for each strain ;^bWT=Wild-type strain

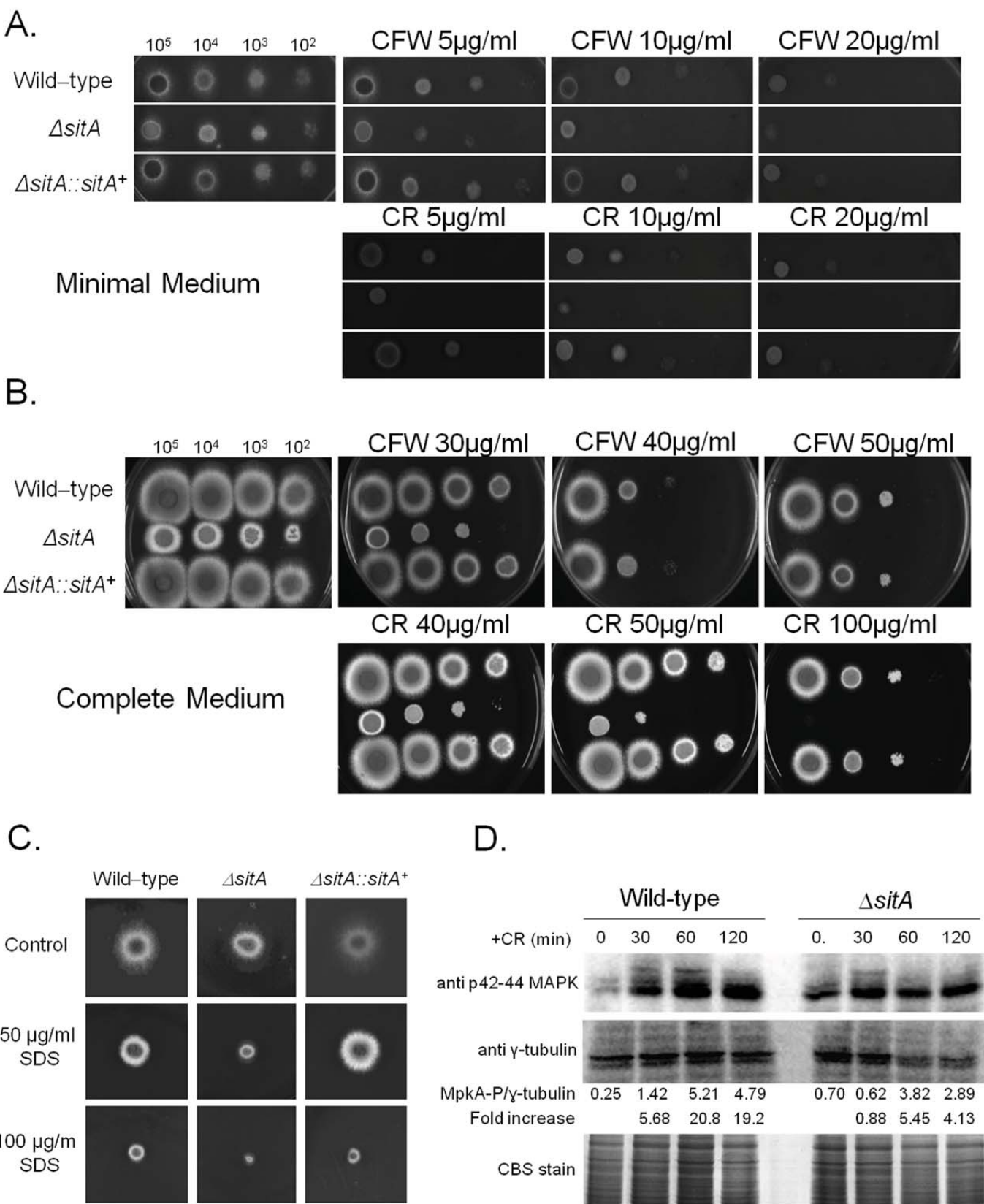
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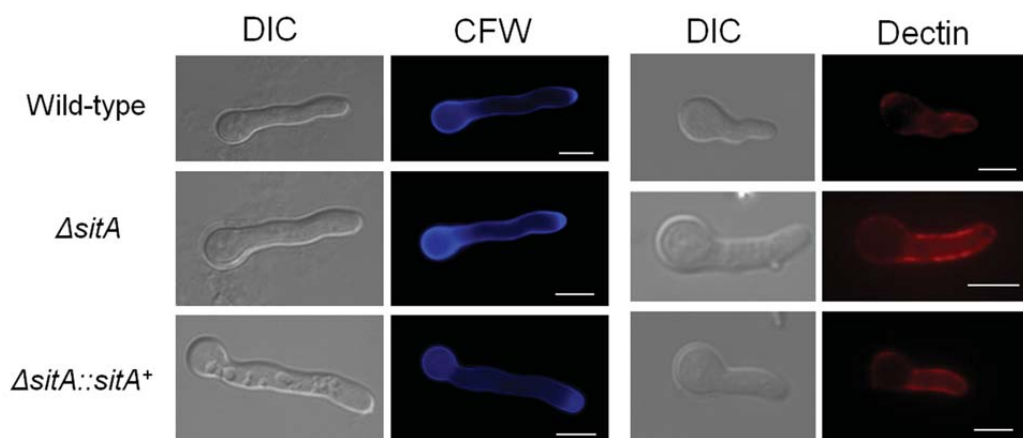
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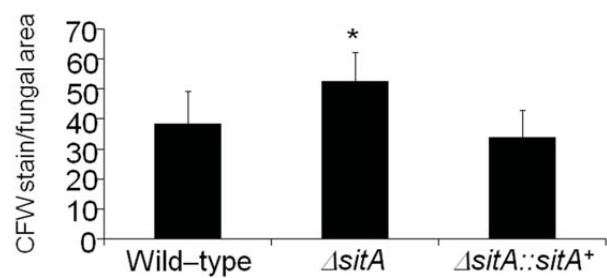




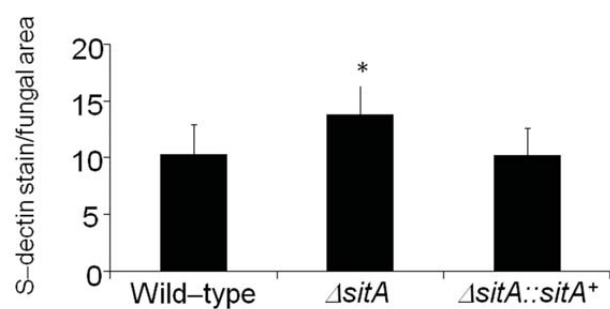
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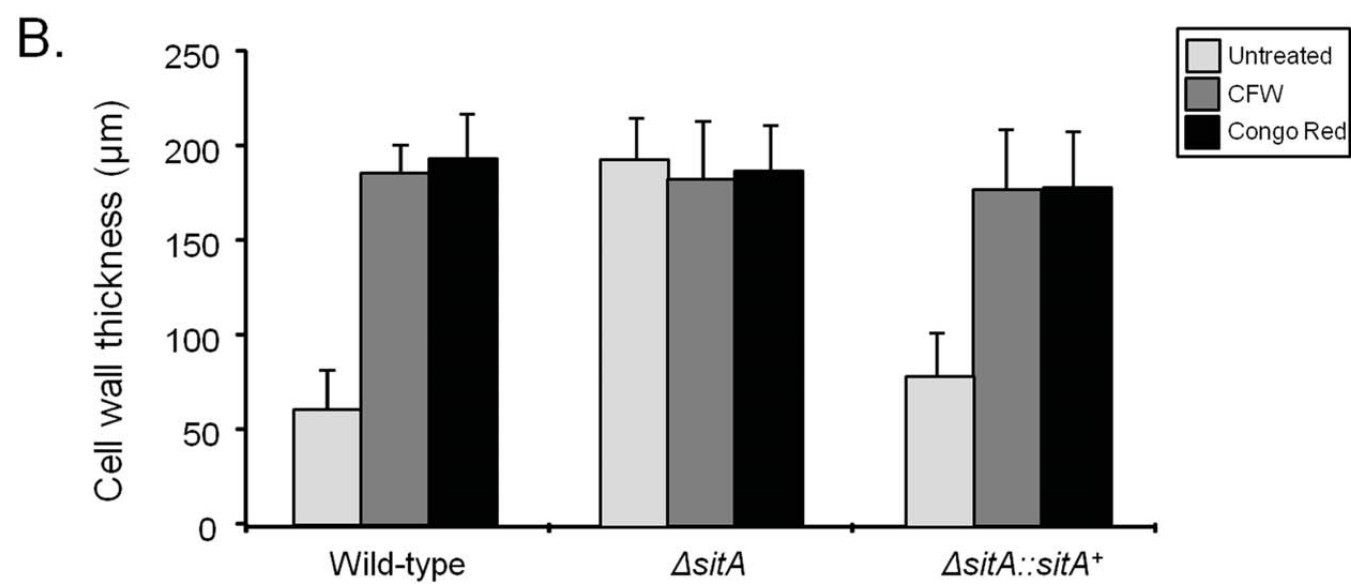
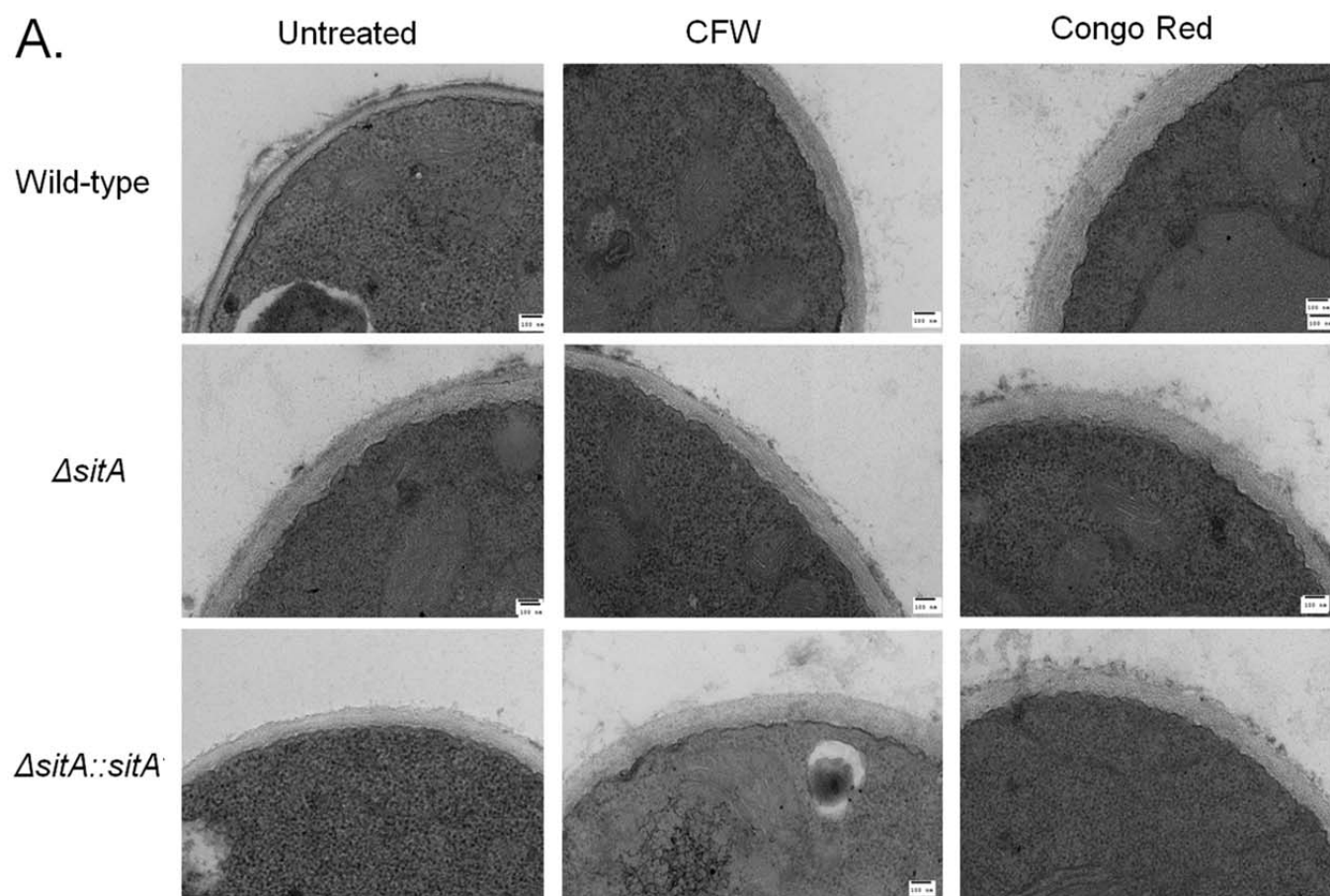


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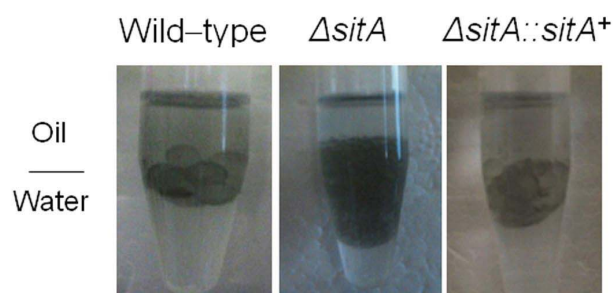


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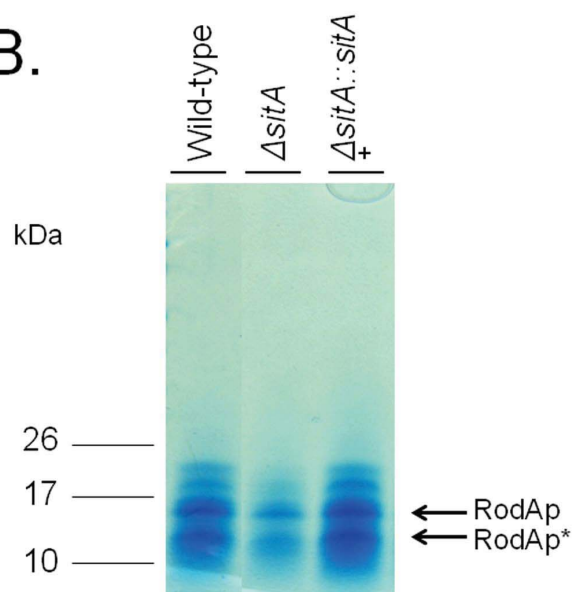




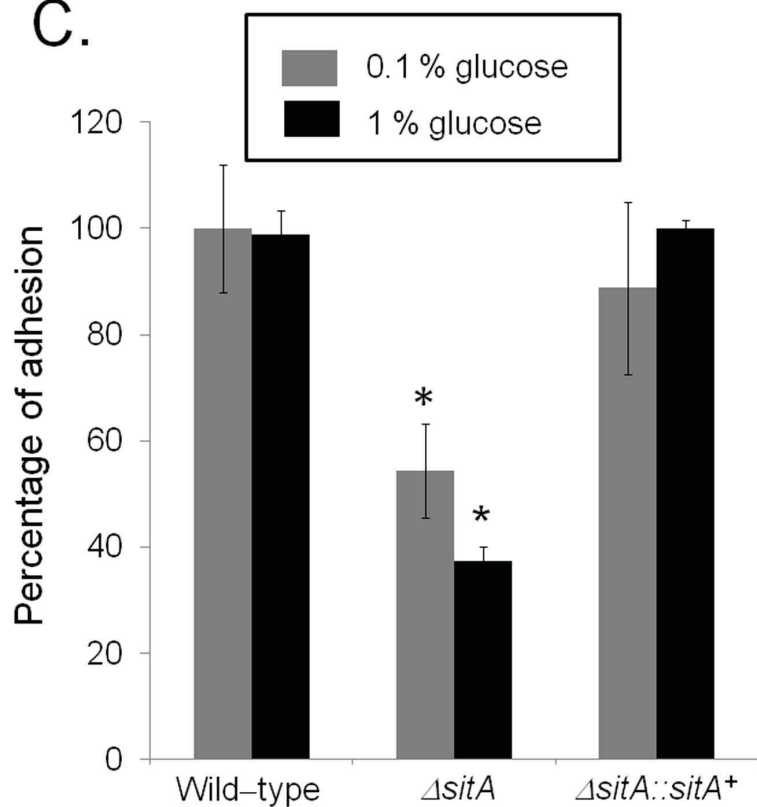
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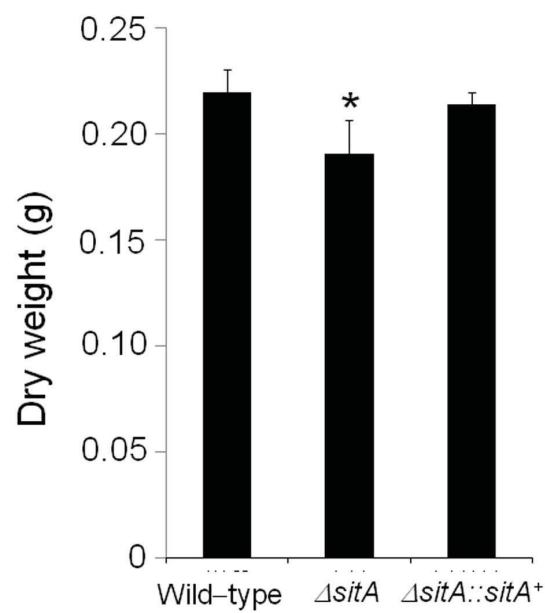
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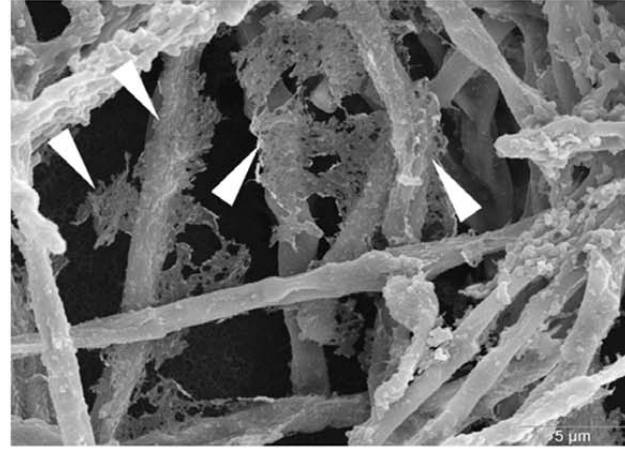
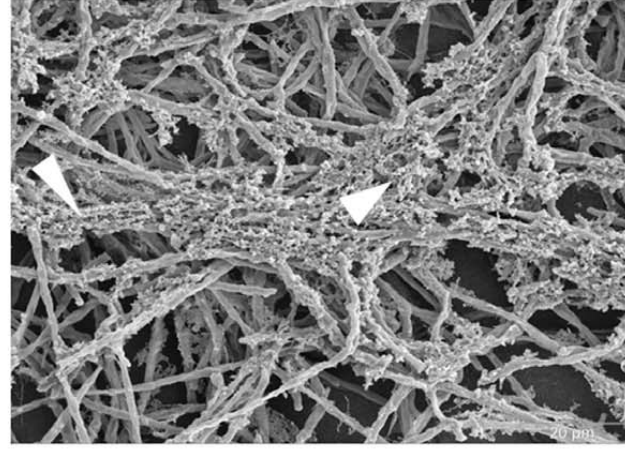
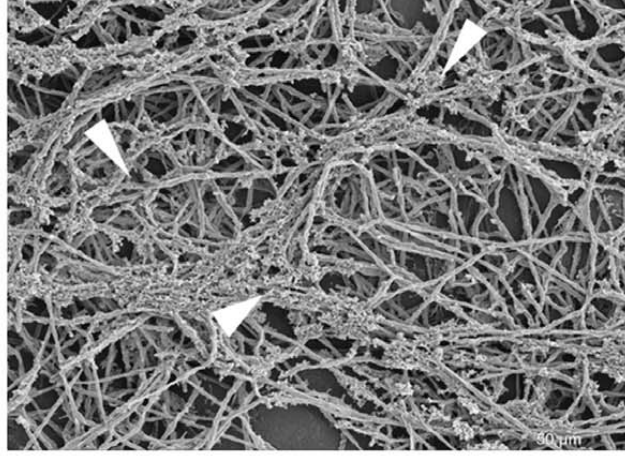
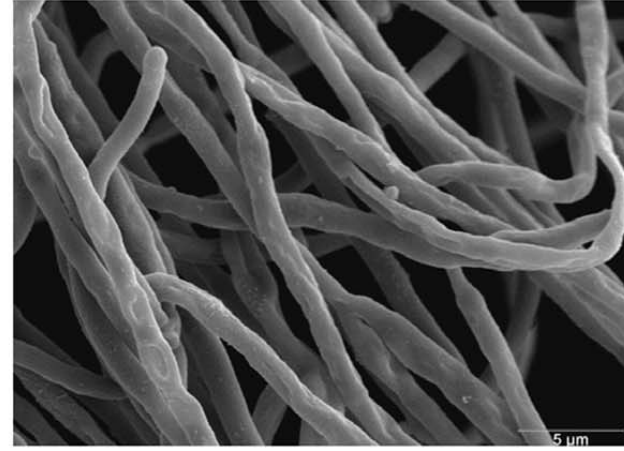
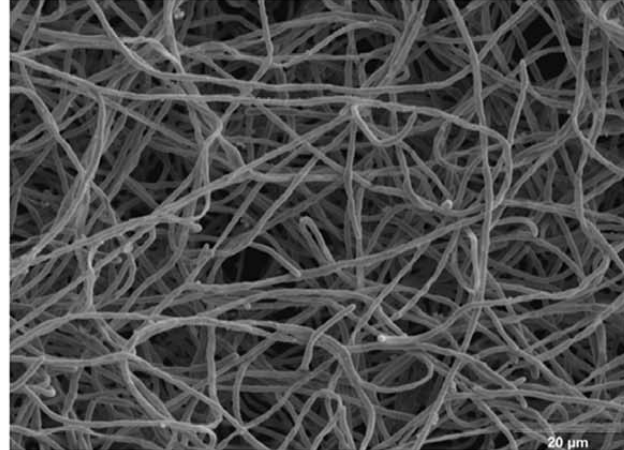
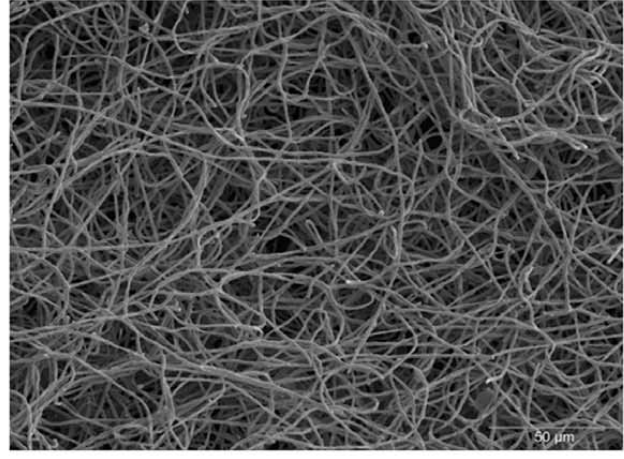
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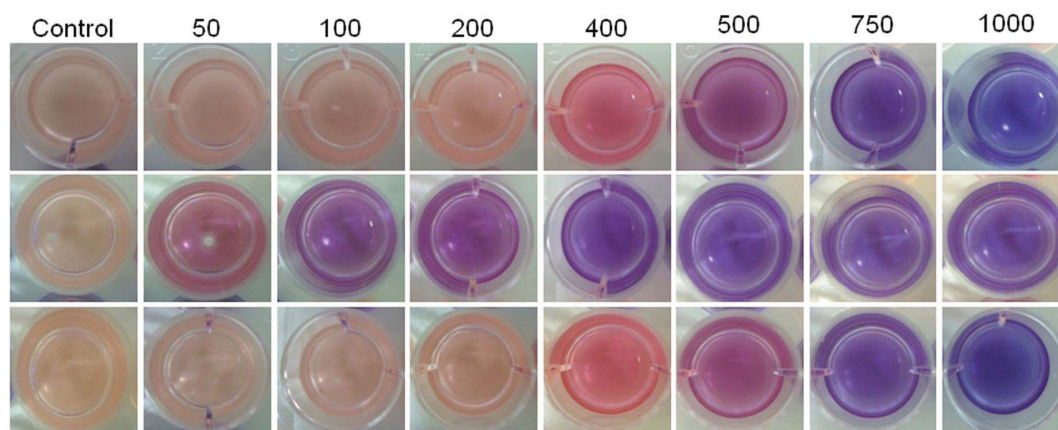
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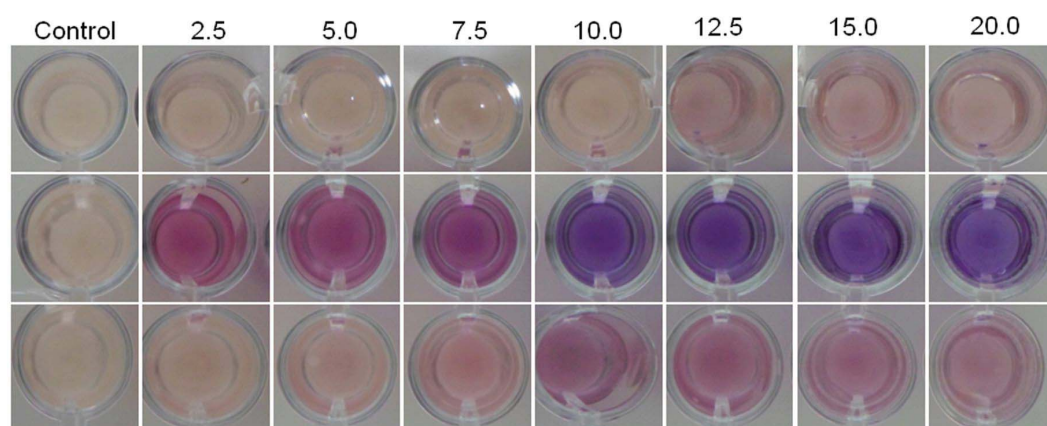
Wild-type

 $\Delta sitA$ 

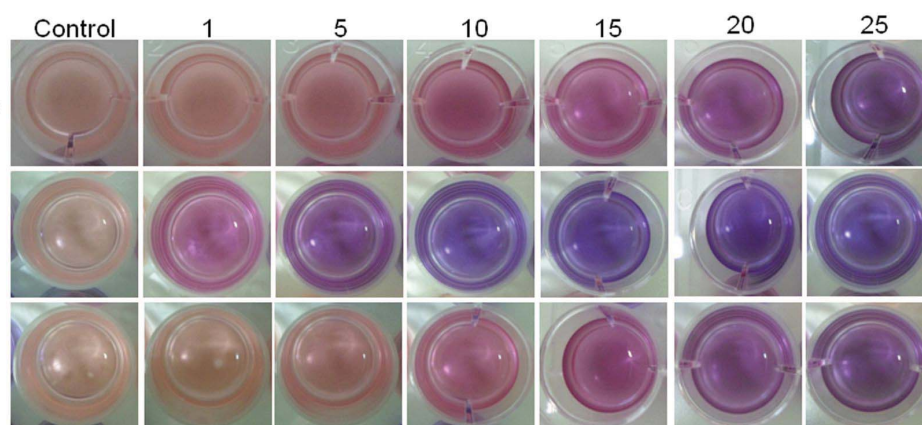
A.

Calphostin C
(nM) $\Delta sitA::sitA$ 

B.

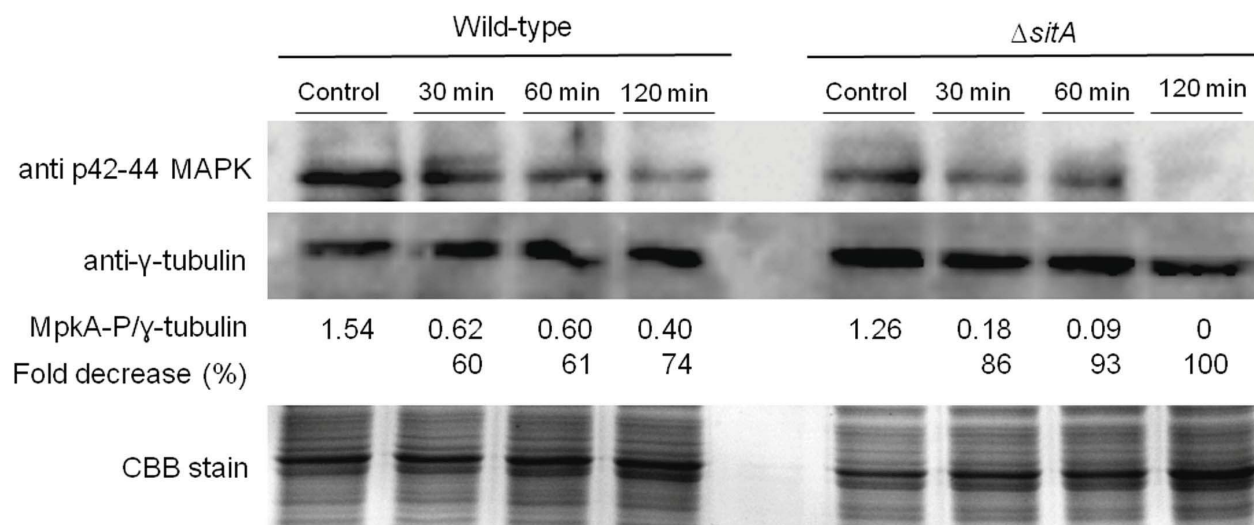
Cercosporamide
($\mu\text{g/ml}$) $\Delta sitA::sitA$ 

C.

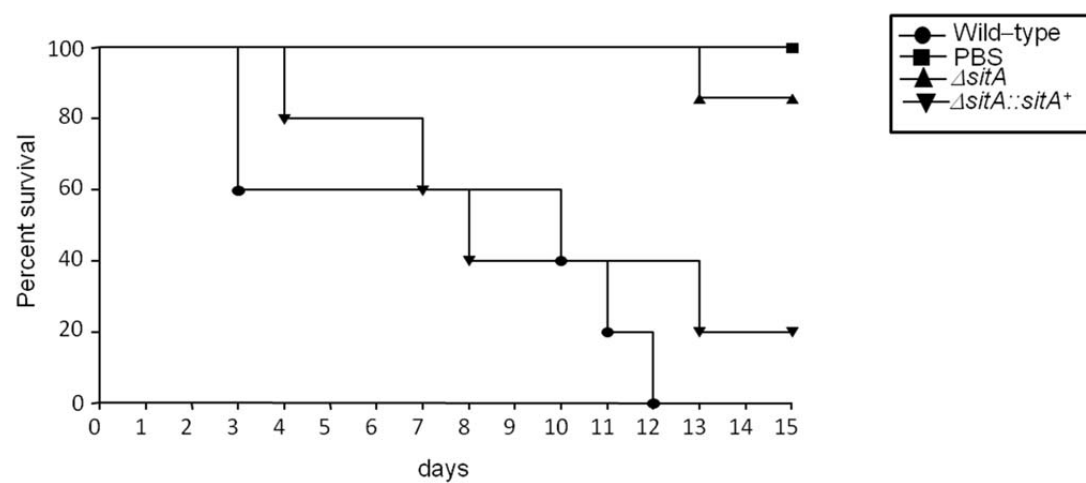
Chelerythrine
(μM) $\Delta sitA::sitA$ 

D.

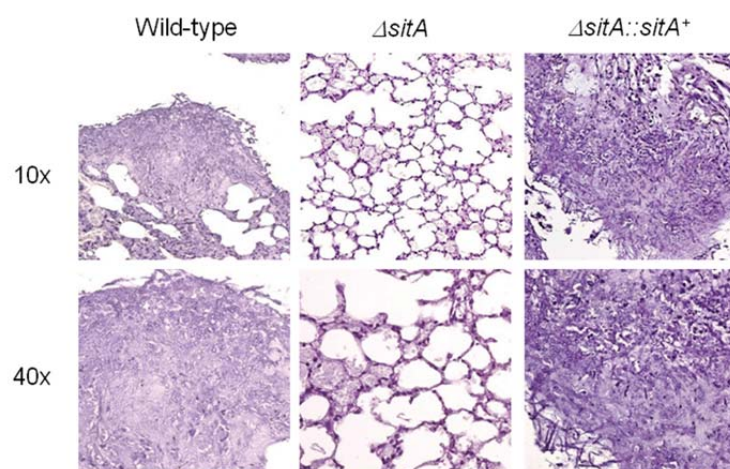
Calphostin C 1000 nM



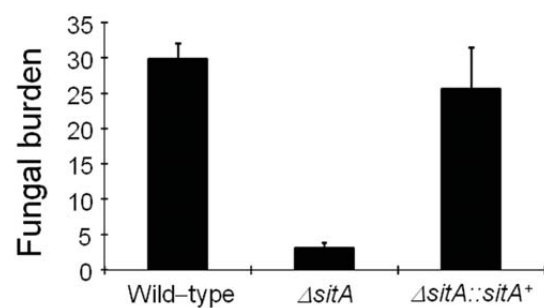
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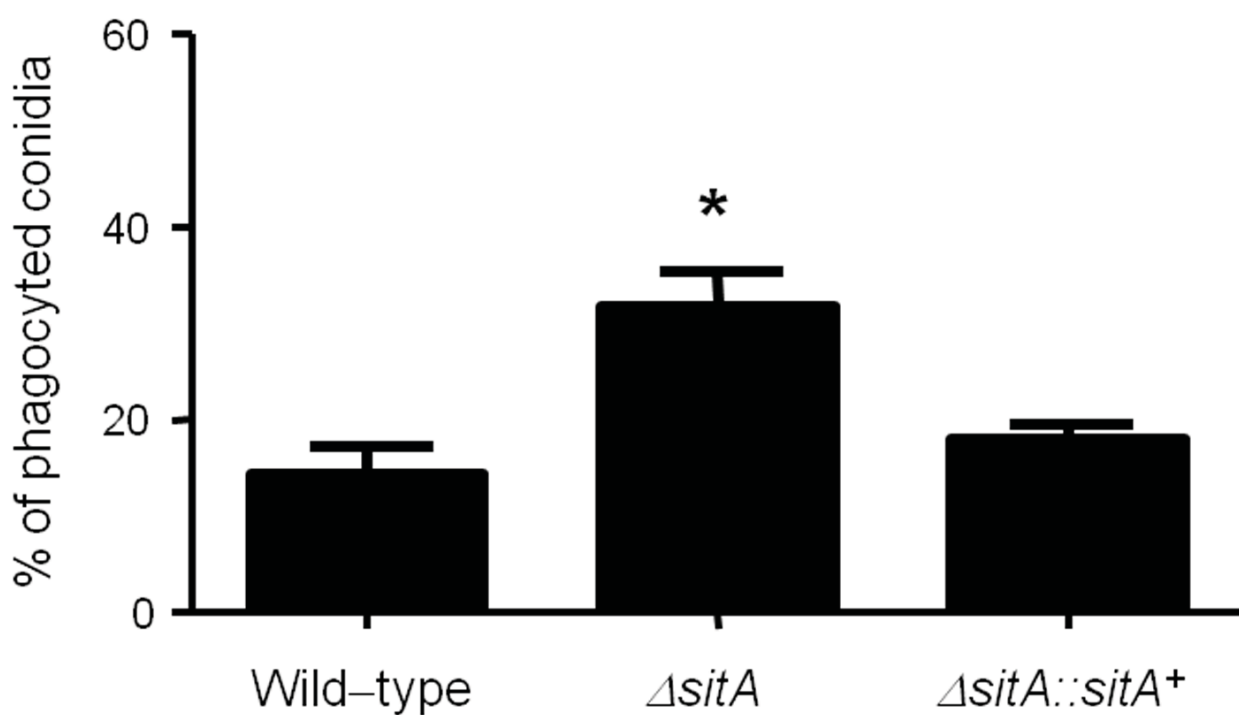
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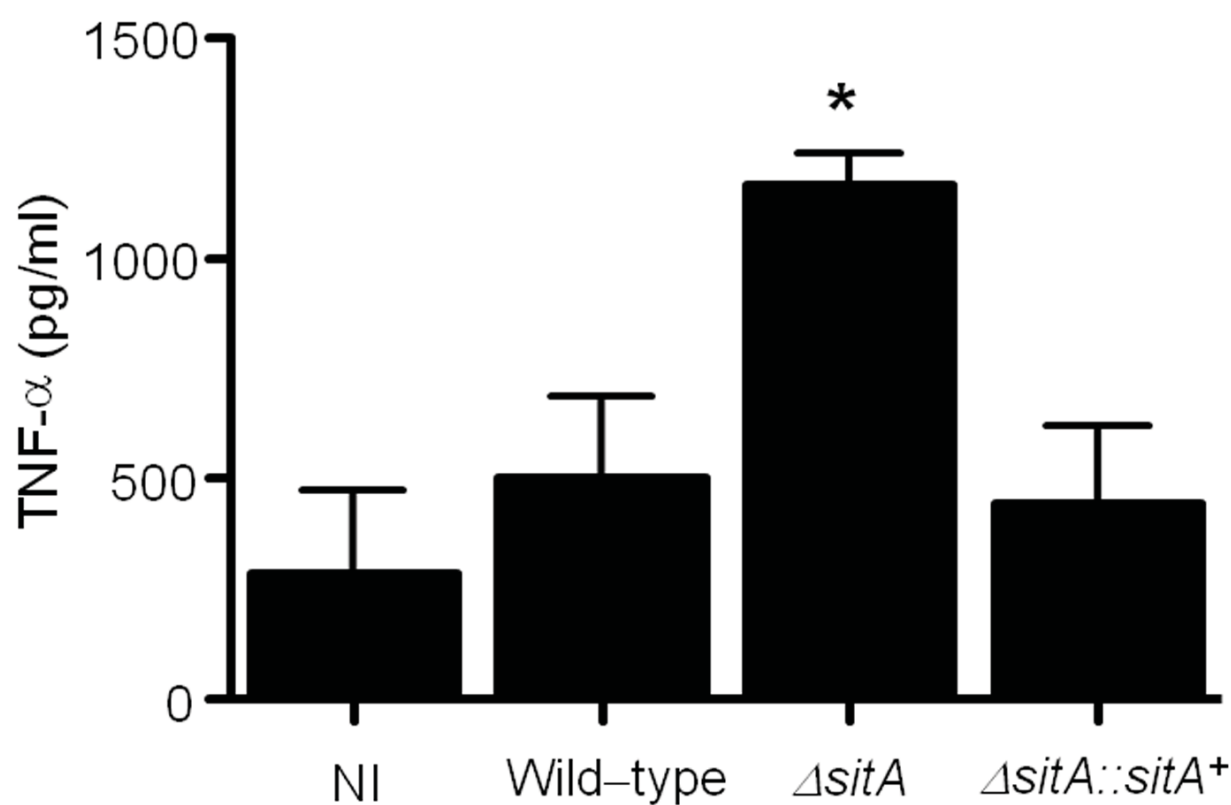
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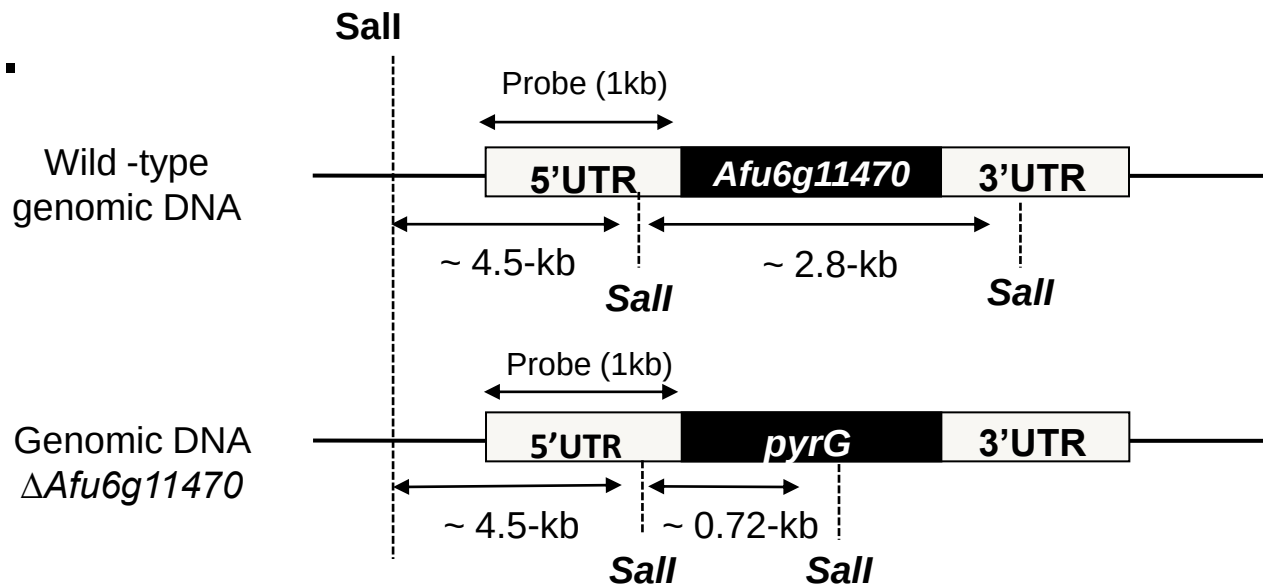
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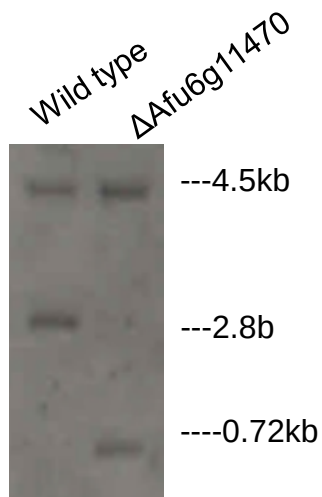
B.



A.



Afu6g11470



B.

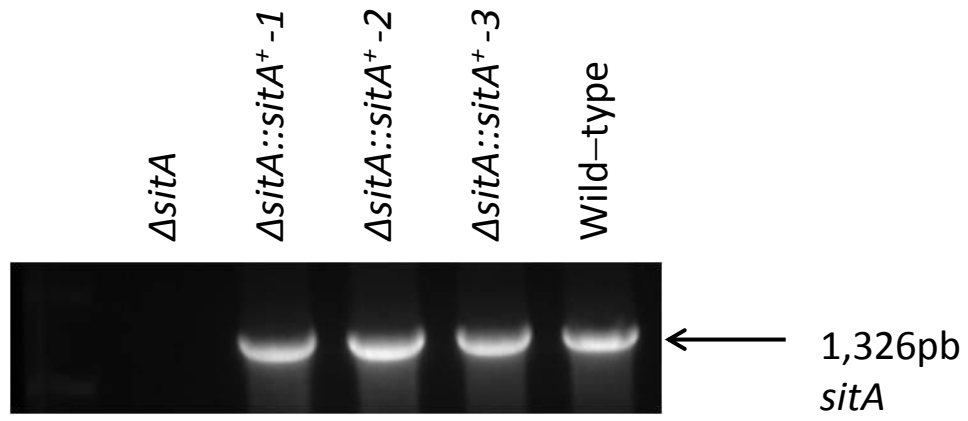
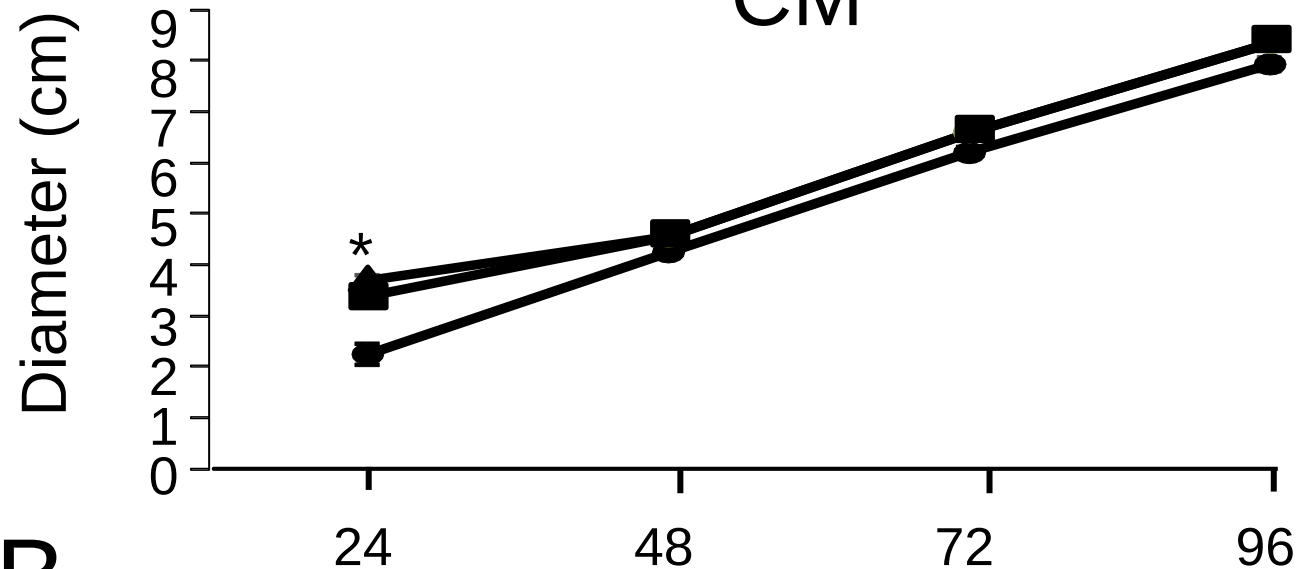


Figure S1 – (A) Southern blot analysis for Afu6g11470 (*sitA*) null mutant mutant.
(B) PCR analysis showing the complementing strain.

A.



B.

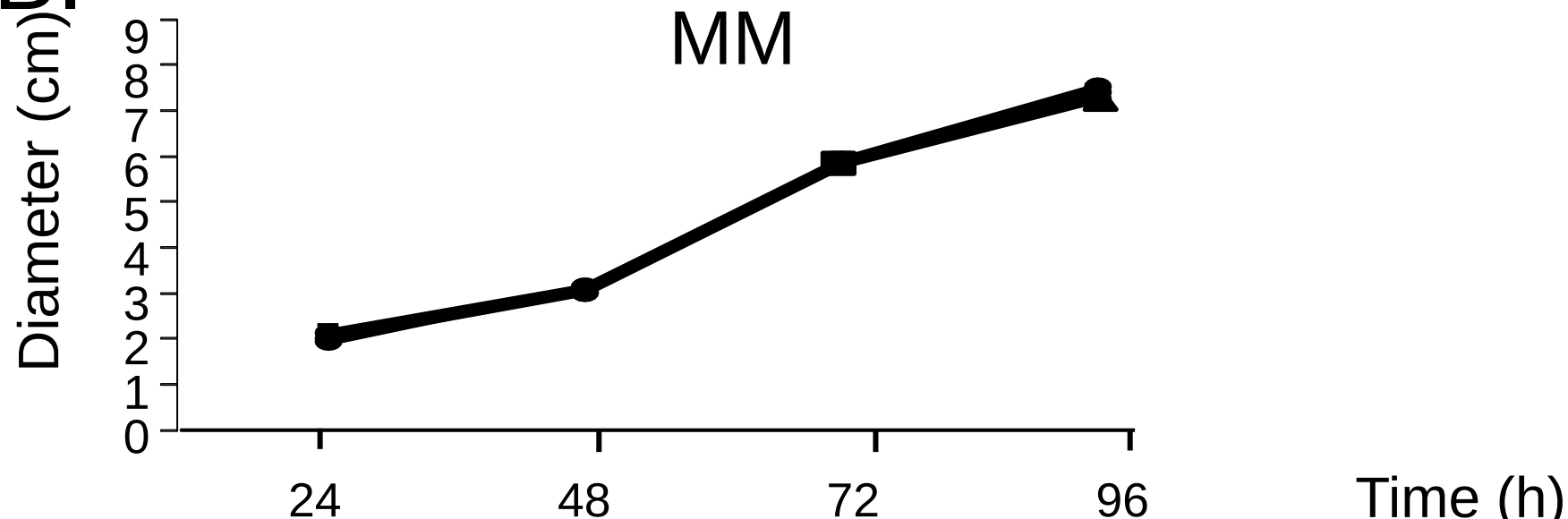


Figure S2 – Growth curves (radial diameter) for *A. fumigatus* wild-type, $\Delta sitA$, and $\Delta sitA::sitA^+$ grown in complete medium, CM (A) and minimal medium, MM (B) for 24 to 96 hours at 37 °C (*, p -value <0.01 when compared to the wild-type strain).

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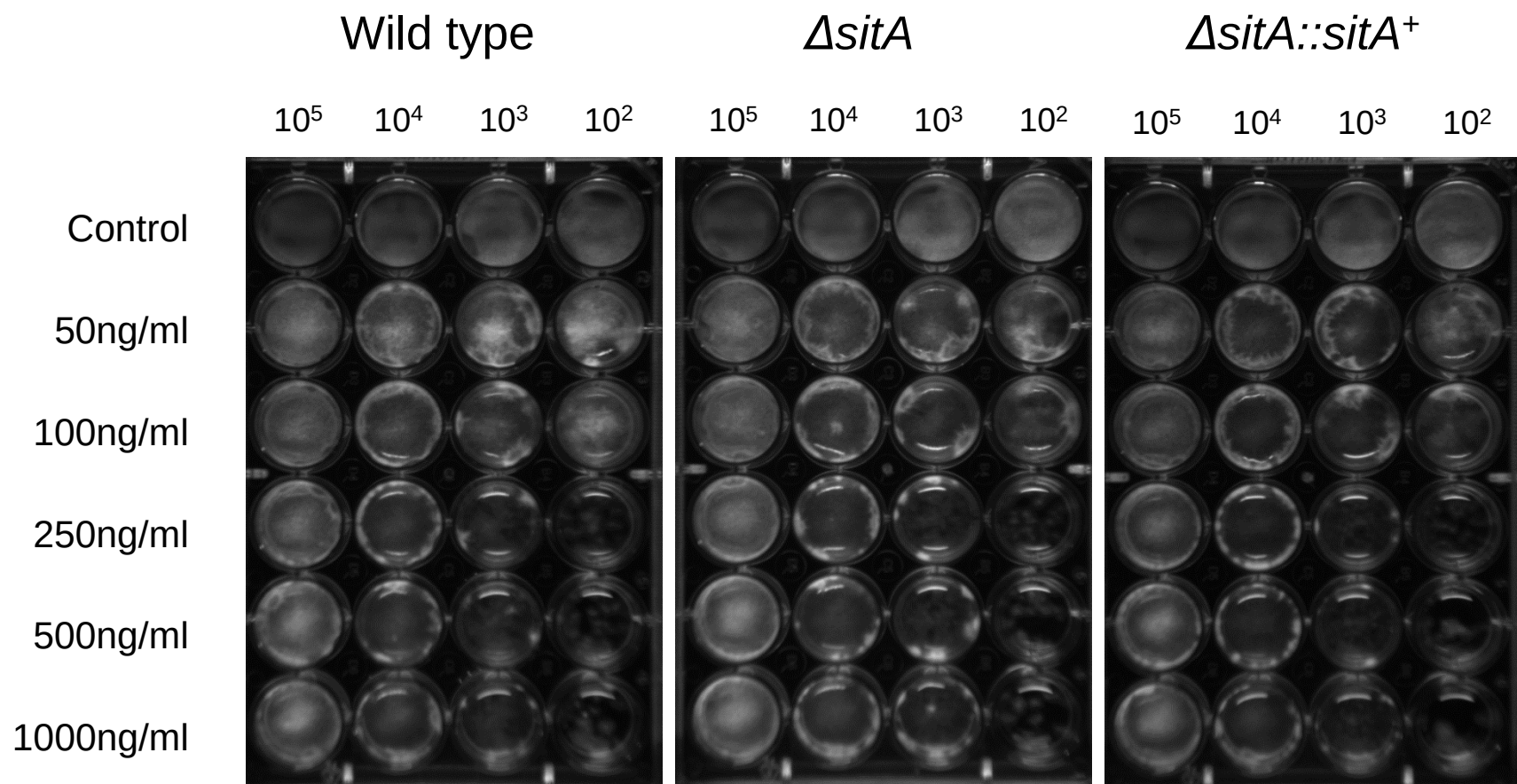
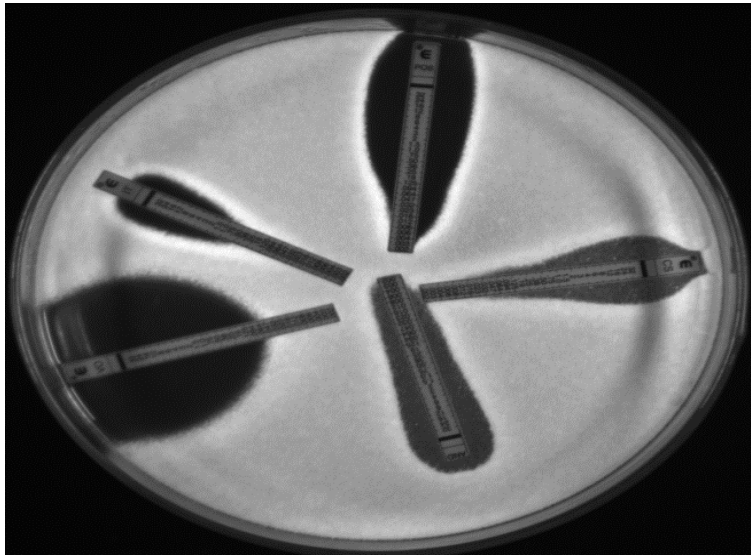


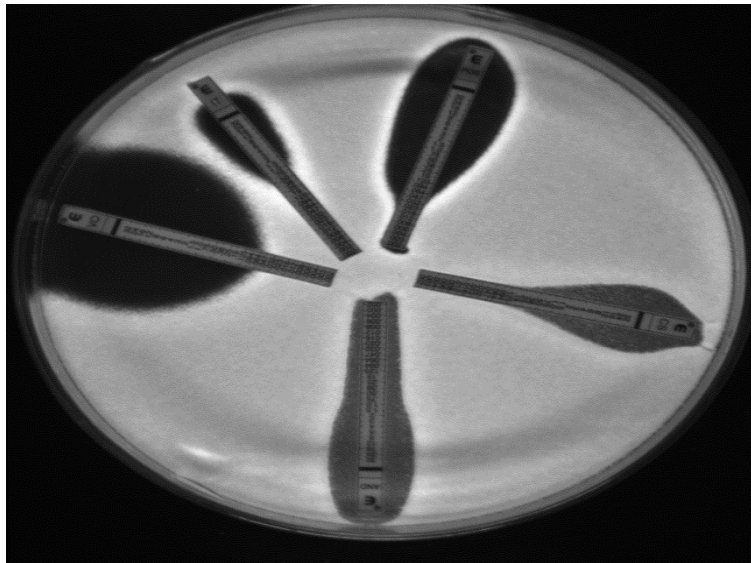
Figure S3 – Conidia dropout growth experiments with rapamycin.

A.

Wild-type



ΔsitA



MIC (μg/ml)	Voriconazole	Itraconazole	Posaconazole	Caspofungin	Anidulafungin
Wild type	0.064	0.75	0.016	0.023	ND
<i>ΔsitA</i>	0.064	0.75	0.016	0.19	ND

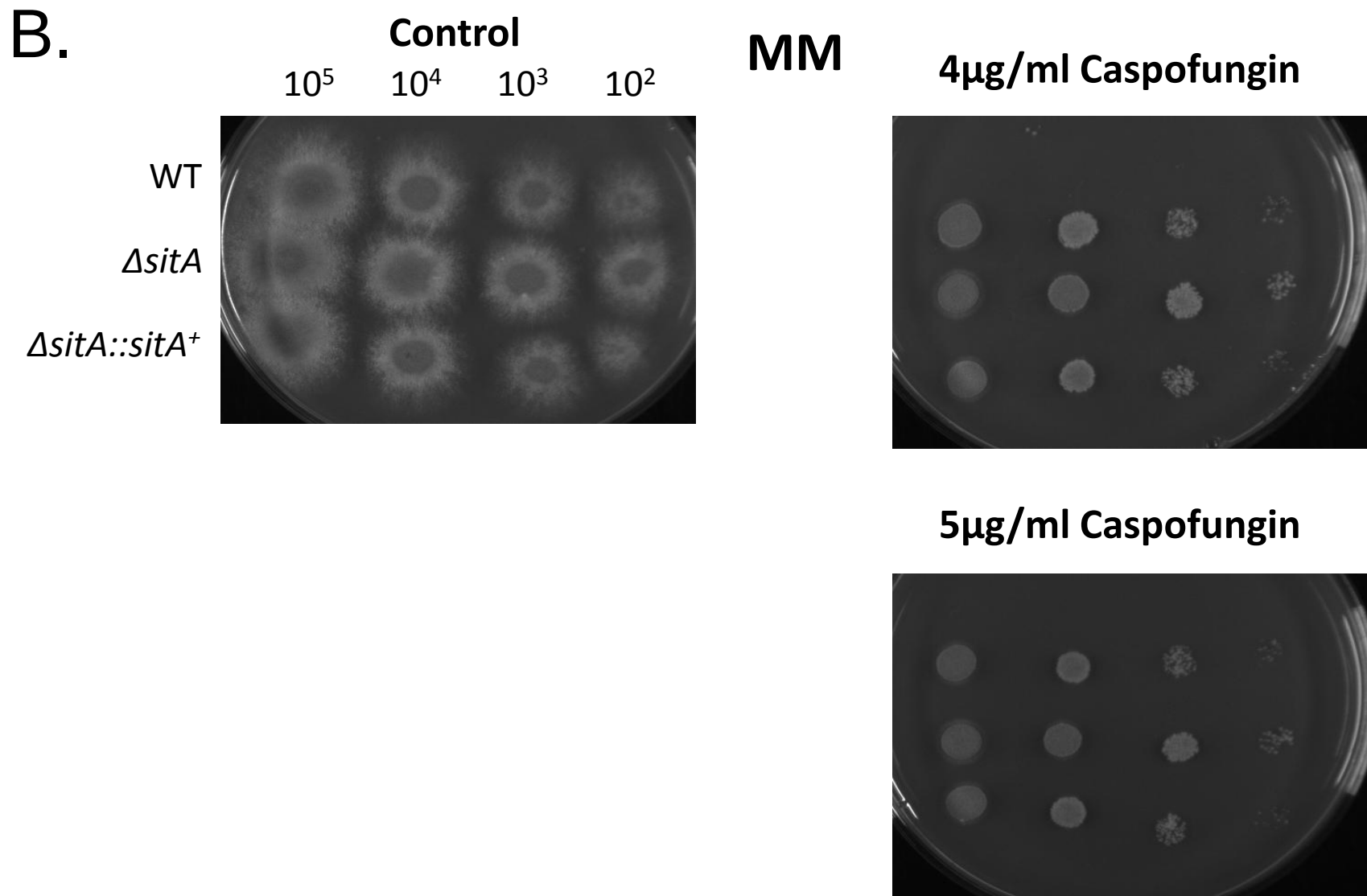


Figure S4 – (A) Etests for the *Aspergillus fumigatus* wild-type and $\Delta sitA$. (B) Drop-out experiments for caspofungin.

Table S1 Oligonucleotide sequences of the primers used in this study

Afu6g11470 pRS426 5fw

5'GTAACGCCAGGGTTTTCCCAGTCACGACGCGGGATCTTGGTCAATTGTACC-3'

Afu6g11470 pyrG 5rv

5'- GCCTCCTCTCAGACAGAATGGTGCGGGTGCGGACGAACG -3'

Afu6g11470 pyrG 3fw

5'- GCATTGTTTGAGGCGAATTCCCTAGCATGCTTGATGGTCTG -3'

Afu6g11470 pRS426 3rv

5'GCGGTTAACAATTTCTCTCTGGAAACAGCCCTGATTATTCCATTCCCGC-3'

Afu6g11470 ORF fw

5'-ATGGTGGACAACAAGATCCCTGAGCCGGG-3'

Afu6g11470 ORF rv

5'-GAGGTATTCTACAAGAAGTACTCGCTGCGGCCCG-3'

Afu6g11470 5'ext fw

5'-CCAGCCCTCCGACGGCCGCC-3'